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SURFACE MEMBRANE CHARACTERISTICS
OF THE HUMAN NATURAL KILLER CELL

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SURFACE MEMBRANE CHARACTERISTICS OF THE HUMAN NATURAL KILLER CELL

By

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SUMMARY

SURFACE MEMBRANE CHARACTERISTICS
OF THE HUMAN NATURAL KILLER CELL

By

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HLA-DR antiserums directed against the allospecific structure of the DR antigen were used in attempts to detect DR antigens on cells responsible for NK activity. Nylon-wool separated PBL were stimulated with either interferon or PHA in an attempt to induce the expression of DR antigen on the NK cell surface if not already present. Data from these studies indicate cells do not normally express DR antigens on their surface, as demonstrated by the inability to abrogate NK cytotoxicity against K562 target cells when effectors were treated with the appropriate HLA-DR antiserum plus complement. Upon stimulation of effector cell populations with interferon or PHA for 18 hours, no appearance of DR antigen on the cell surface was detected. High levels of activated lymphocyte killing (ALK) were observed in PHA stimulated cells after only 18 hours of culture. This increased cytotoxicity against K562 cells was not diminished by treatment of cells with the appropriate HLA-A or B locus antiserum plus complement. On the basis of this and previous studies it is concluded that the effectors of natural killing do not normally express DR antigen on their surface and cannot be induced to do so by stimulation.

To

my parents

Without their constant love and support

neither this thesis nor any of the goals

I have achieved thus far in my life would have been possible.

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INTRODUCTION

The cytotoxicity mechanism shown by NK cells has drawn much attention since these cells were first recognized as possibly playing a role in homeostasis. Although much effort has been directed towards identifying surface membrane structures involved in regulating NK cell cytotoxicity, there has not yet been any identification of antigens directly responsible for target cell killing. In studies of the cytotoxic mechanisms employed by T-lymphocytes, the HLA-D(DR) gene products in humans have been shown to be the primary stimulatory antigens for the generation of cytotoxic effector cells in mixed lymphocyte reactions (73). The HLA-DR locus or a closely linked locus appears to correspond to the immune response (Ir) genes found in the murine system. This region of the genome has been attributed with controlling the immune system's response to antigenic challenge as well as being correlated with susceptibility to certain diseases (87-89). Since the level of NK activity in humans and animals appears to be gentically determined and may be linked to genes of the major histocompatibility complex (90,91) it is logical to look for DR gene products on the NK cell surface. Until about 1977 human DR antigenic determinants were thought to be expressed only on surface immunoglobulin bearing lymphocytes and monocytes (92,93). In 1977 expression of DR antigens was found to occur on 72 hour mitogen stimulated T-cells (94). This present study was performed in order to determine whether DR antigens are normally expressed on NK cells or if their expression could be induced by stimulating the cells with interferon or mitogens.

LITERATURE REVIEW

Immunologic Surveillance and the Natural Killer Cell

In 1963 Burnet used the term immunological surveillance to describe "the concept that a major function of the immunological mechanisms in mammals is to recognize and eliminate foreign patterns arising in the body by somatic mutation or by some equivalent process" (1). Burnet postulated that if the concept of surveillance was correct it should be possible to show the facilitation of spontaneous tumor appearance or successful adoptive transfer of tumor cells by neonatal thymectomy. This hypothesis necessarily attributed almost sole responsibility of immunological surveillance to the thymus-dependent system of immunocytes (2). Skeptics of this surveillance hypothesis opposed the designation of tumorigenesis as the main purpose for the existance of the cellular immune system. It was their opinion that the daily microbiological stress of infectious agents would be much more probable justification for the maintenance of the cellular immune system against evolutionary pressures.

The early data demonstrating increases of malignant disease in neonatally thymectomized or immunosuppressed individuals were interpreted in favor of the surveillance hypothesis (2). Opponents though were later given support for their viewpoint when nude mice were used in malignancy studies. Nude mice and normal mice showed no difference in tumor incidence due to treatment with chemical carcinogens such as 3-methyl cholanthrene administered at birth. Latent periods for tumor development were also comparable (3). The opponents of the tumor surveillance hypothesis cited this as evidence to refute Burnet's hypothesis on the

function of thymus dependent lymphocytes. The subsequent discovery of a population of cells that matured independently of the thymus, but with the ability to kill tumor cells, has lead to some modification of the tumor surveillance hypothesis.

The concept of immune surveillance should not be taken strictly with regard to any one cell population being solely responsible for tumoricidal activity. Rather, surveillance becomes more acceptable when looked at as a phenomenon which involves all categories of immune cells in an effort to maintain homeostasis. The one cell type which most closely satisfies Burnet's original definition of an immune surveillance function is the natural killer cell. It is this cell population that is believed responsible for the low incidence of spontaneous tumors in nude mice. NK cells are able to act before a conventional immune response can be mounted against abberant cells (4). They are present in animals with no known previous exposure to the antigens necessary to induce killer T-cell subsets. These facts along with a rapid ability to carry out their effector function lends support to this cell population serving as the first defense barrier against neoplastic growth (5).

Antitumor Role of NK Cells

Considerable attention has been given to the phenomenon of nonspecific killing of tumors. Originally investigators were concerned with specific cell-mediated immunity to human cancers. Hellstrom and others (6-10) demonstrated specific killing of tumor targets of the same histiologic type as the patient whose lymphocytes were used as effector cells. Killing of tumor cells by lymphocytes of normal healthy controls was ignored until Takasugi et al. found lymphocytes from normal persons were as reactive or more reactive than those from cancer patients that had the same type cancer as the derivation of the target cell (10). Both

cancer patients and normal individuals were found to vary in reactivity against each target culture. The implication of this was that cell mediated lympholysis (CML) was not limited to tumor specific anitgens. DeVries et al. compared cytotoxic effects of cells from melanoma patients and healthy donors on short term tumor cell cultures and established cell lines (11). They found that lymphocytes from healthy donors showed cytotoxicity to more cell lines than short term cultures while lymphocytes from melanoma patients reacted well with both short term cultures and cell lines. In fractionating the lymphocytes into T and non-T cell populations the cytotoxic effects of healthy and melanoma subjects were recovered in non-T-cell fractions. The fractionated non-T-cell populations of melanoma and healthy subjects reacted as strongly or more strongly than total lymphocyte populations. Tumor cell killing not mediated by specific tumor associated antigens was concurrently described by others (12-16). The concensus of all these experiments was that nonspecific killing in normal healthy donors was as strong or stronger than killing from cancer patient cells. Recognition of significant reactivity among normal control lymphocytes was important since the extent of cytotoxicity displayed by cancer patients' lymphocytes is often expressed in comparison to control values (16).

The observation of nonspecific killing by natural killer cells has modified the manner in which cancer patients' immunocompetance can be assessed. In studies of acute myelocytic leukemia patients in remission, mononuclear cell preparations have spontaneous cytotoxicity values similar to or higher than those of normal control subjects. Heavy combined chemotherapy will substantially reduce the killing of K562 target cells by lymphocytes of AML patients. Furthermore, a drop in NK function of patients in complete clinical remission has been found to be a reliable early warning of an impending relapse (17).

Murine studies have found that metastatic tumor cells are more resistant than cells of local tumors to killing by NK effectors (18). This difference has been interpreted as a limitation of the extent to which NK cells can control tumor spread. By defining the role and functions of NK it is assumed that this information can be applied to early detection and control of spontaneous tumors.

Anti-Graft Role of NK Cells

The role of NK cells in hemopoietic graft rejection has been extensively examined for similarities with the effector cell population(s) and regulatory mechanisms responsible for this type of graft rejection. The most informative findings have come from murine models. Hemopoietic resistance in irradiated mice leads to the rejection of normal and malignant transplanted parental, allo and xenogeneic hemopoietic grafts, but not other tissue grafts. The cells responsible for hemopoietic resistance differ significantly from those cells which are responsible for the classic transplantation reaction by several characteristics. These characteristics include: resistance to high amounts of total body irradiation; tissue specificity restricted to hemopoietic tissues and their malignant derivatives; prompt reactivity (rejection as early as 18-96 hours after transplantation); late maturation (about 3-4 weeks after birth); thymus independence and no need for presensitization (19). These characteristics are also shared by natural killer cells.

It has been observed that treatments such as 1100R whole body irradiation, cyclophosphamide, silica, t-carragenan and C. parvum, which made mice tolerate bone marrow grafts, also drastically reduced NK levels (19-23). Silica and t-carragenan have a marked effect on reduction of natural killing. These two agents are also believed to be specific inhibitors of macrophage and it is therefore possible that macrophage play some role, either directly or through extracellular mediators, in the NK cell's participation in marrow cell rejection.

The association between pre-transplant NK levels and graft-versus-host disease after stem-cell transplantation has been studied in humans (21). NK activity against herpes simplex type 1 infected fibroblasts (NK-HSV-1) was determined in patients prior to undergoing bone-marrow or fetal-tissue stem-cell transplantation. All patients surviving the grafts with no evidence of graft-versus-host disease were those with low NK (HSV-1) levels prior to transplantation.

In examining shared characteristics of NK effectors and effectors of hemopoietic graft rejection Hochman et al. found different sensitivities to hydrocortisone of the two effector populations (20). NK activity was sharply decreased after in vivo drug administration whereas induction of specific F_1 anti-parent or anti-allogeneic cytotoxicity and hybrid resistance to parental marrow grafts was not impaired. Considering the large number of other shared properties Hochman postulates that NK cells and cells responsible for anti-graft reactivities are probably generated from a single differentiation pathway, but are not the same cell, differing with respect to specificity of targets and sensitivity to hydrocortisone (20).

Cytotoxic Mechanisms of NK Cells (Mode of Killing)

Although the exact mechanism(s) involved in the NK cell mediated lysis of tumor targets is still unknown, some of the physical characteristics have been described. However, these descriptions are not without controversy. It had been shown that target cell binding by the NK effector cell was a prerequisite to killing (24,25). Trypsinization of nylon-wool passed effector cells reduced both binding and lysis. Lysis regenerated in a parallel time course with binding after incubation at 37°C. Target binding was also found to occur over a broad range in temperature, with a plateau reached in 20 minutes at 37°C compared to 40 minutes at 0°C. Nylon-wool adherant cells bound to targets immediately after centrifuga-

tion together and showed no increase of binding with time. This contrast with nylon-wool nonadherant cells suggests that binding of the NK effectors occurs by a different mechanism and has some specificity (24). Lysis was found to lag 5-10 minutes behind the first increase in target binding. While binding did occur over a broad range in temperature, lysis did not occur at 15°C, and lysis at 20°C was only half of that which occurred at 37°C.

Binding and lysis have been shown to be independent by methods used to inhibit one mechanism and not the other. EDTA inhibits cell contact and therefore, lysis, by removing the divalent cations which are necessary for cell binding. Regeneration of binding after trypsinization was inhibited by incubation with cycloheximide. This suggests that de novo protein synthesis is needed to regenerate target cell binding sites on effectors. Metabolic inhibitors such as DNP, NaN_3, inhibitors of serine proteases or inhibitors of microtubules (colchicine) inhibit lysis, but have no effect on the frequency of target cell binding. All these treatments suggest membrane associated cytoplasmic changes are involved in lysis (24).

All previous attempts to find a soluble mediator involved in NK-sensitive target lysis have been unsuccessful. Effector-target contact was deemed an absolute prerequisite for lysis to occur. Recently, soluble mediators released from murine spleen cells stimulated with PHA or CON A have been found to cause target cell lysis after a 40 hour incubation period (26). Significant lysis of NK targets was not found before 40 hours of incubation. The killing caused by this soluble mediator was shown not to be due to nutrient depletion or other culture conditions of the targets. The same mediator dependent killing was found in the human system using K562 cells as targets. Irradiation of target cells to prevent replication of DNA did not affect the results, indicating that target lysis does not result from inhibition of DNA synthesis. Since the assay time was 40 hours before

mediator induced lysis was shown to occur and NK lysis against K562 is highest in rate during the first hour, decreasing to almost zero after 4-6 hours (26), it is unclear whether this soluble mediator takes part in the lytic mechanism responsible for target death.

Through isolation of the target binding cells responsible for natural cell mediated lysis the effectors were found to be a large lymphocyte with a granular cytoplasm. These cells had a strong cytoplasmic acid-α-napthyl-acetate esterase (ANAE) reaction. This is also characteristic of a T-lymphocyte (25). Another characteristic which is apparent on all NK effectors (most noticeably on human cells) is the receptor for the Fc portion of IgG. A major area of controversy exists over the role of the Fc receptor in NK cytotoxicity. It has been proposed that natural cytophilic antibodies bind to Fc receptors on NK cells and arm them specifically for their target cells. Attachment to targets then occurs at antibody binding sites and lysis follows. This mechanism of action has been supported by studies showing that effector cells treated with trypsin lose their cytotoxicity (27,28). Trypsin is thought to shear off the natural cytophilic antibodies from NK cells. The cytotoxicity can be restored to these cells by incubating the lymphocytes in autologous serum. Unfortunately, this has not been substantiated in studies utilizing tumor cells or fetal fibroblasts even with prolonged incubation in autologous serum (29-31). Treatment of lymphocytes with trypsin destroys the NK activity while leaving levels of ADCC unaffected. Since ADCC requires intact Fc receptors in order to operate, trypsin treatment of lymphocytes must not destroy these receptors (30). Incubation of NK effectors at 37°C for one hour after trypsin treatment allows regeneration of control NK levels. Pronase treatment however, destroys both NK and ADCC activity. An 18 hour, 37°C incubation allows full recovery of both these activities. When lymphocytes are incubated with immune complexes a reduction in NK activity is seen which cannot be regained after

prolonged 37°C incubation. All these findings suggest that NK activity requires some recognition structure(s) which act alone or in conjuntion with the Fc receptor, are sensitive to proteolytic enzymes and can be resynthesized in culture.

It has been shown that NK cells are a heterogeneous population with respect to the target cell antigens they recognize (32,33). Through target cell monolayer adsorption studies, subpopulations of NK cells have been separated from a whole population of lymphocytes. It has not been clearly shown whether a single subpopulation of NK cells can recognize only one target specificity, or if receptors for more than one type of target structure exist on individual NK cells. If there are receptors for more than one target structure on an NK cell, a question is raised about whether binding can occur with equal strength at any receptor or if certain NK cells show a preference for binding one target structure over all others. Callevaert et al. showed in competetive binding assays that cross-inhibition of NK cell lysis can occur using an inhibiting cell population different from the labeled target cell (34). This indicates that certain target cell lines do express the same or similar sets of target antigens recognized by NK cells. However, most cells also contained unique target antigens, as shown by the fact that little or no inhibition could be obtained by any other cell line than an unlabeled cell homologous to the ^{51}Cr labeled target.

One last question to be addressed in a discussion of the lytic mechanism of NK cells is whether NK cells are inactivated after target cell binding or does recycling occur? Using a cloned K562 cell line unable to induce interferon production when co-cultured with lymphocytes, Perussia et al. showed that NK cells are inactivated after binding with their target cell and were unable to go on to kill other cells (35). However, with all target cell lines able to induce interferon production there was no inactivation of NK. Perussia obtained his data using a single NK-target cell assay in which cells were immobilized in agar. Single, dead

target cells unattached to effector NK cells were interpreted as physical evidence of recycling. Inactivation was time and temperature dependent and did not affect the ability of lymphocytes to execute ADCC. Lymphocytes which were incubated with target cells and then separated by physical means had a decreased lytic ability compared to lymphocytes not undergoing this treatment. Upon incubation with interferon their cytotoxic activity was restored. Although binding of targets occurred (at varying rates) at different temperatures, lysis of targets only occurs at 37°C. Complete lytic inactivation of the K562 clone occurred only with lysis of a target. These facts and the role of interferon suggest inactivation occurs due to activation of the NK cell lytic mechanism or possibly by an alteration of a surface receptor after cell-cell contact.

In studying the recycling capacity of NK cells against targets Ulberg and Jondal examined individual effector-target cell conjugates and showed that a smaller fraction of NK cells had killed their target than those NK cells bound to other target cell types (36). However, since ^{51}Cr release assays using K562 targets displayed as high or higher values of radioactive counts than when other targets were used, the conclusion was that there existed a high amount of recycling ability for NK cells against the K562 cell line. There were discrepancies between the percentage of target binding cells and the overall level of lysis. This further supported the idea that not all lymphocytes that bind to targets go on to lyse them and recycling is needed to explain the levels of lysis present.

Surface Membrane Characteristics of NK Cells

A large amount of evidence now seems to indicate that the effector cells for NK are of the T-cell lineage, although they apparently must be a subpopulation since they develop independently of the thymus (37). Initial discrepancies between investigators of human NK cells about surface receptors such as complement, the

Fc portion of IgG, sheep erythrocytes and sensitivity to specific antihuman T-cell serum plus complement appear to be due to technical differences. Use of optimal conditions for E rosette formation showed the majority of human NK cells to have receptors for erythrocytes (37). Mouse NK activity has been eliminated by treatment with high concentrations of anti-Thy 1 serum plus complement (38). Low density Thy 1+ cells have been described in nude mice (39,40) and suggest that those cells are pre-T-cells (37). The concept of NK cells being at an early phase of maturation in the T-cell lineage is also supported in work (41,42) where incubation of mouse or human lymphocytes for two hours with certain thymic hormone preparations associated with mature T-cells, leads to decreased NK activity (37). Fc receptors are present on NK populations in mouse, rat and human populations, but are difficult to detect on all but human NK cell populations. No surface membrane immunoglobulin receptors have been detected on NK cells and no NK cell populations have been found to adhere to nylon-wool or be phagocytic (37). Complement receptors are undetectable on the majority of NK cells (43,44). Pross et al. showed that complement receptors may be present on, at most, one-third of human NK cells (43). West et al. speculated that some reports of complement receptors on human NK cells may have been due to the presence of IgG antibodies in the antisheep erythrocyte reagents, causing rosette formation through the Fc-IgG receptor (44).

Several important differences were found in surface properties of activated NK cells versus endogenous NK cells. Kiessling et al. compared the cell surface properties of nonactivated "endogenous" NK cells from normal mice with NK cells activated in vivo by acute infection with lymphocytic choriomeningitis virus (LCMV) or in vitro by interferon (45). Some major differences between the populations were: 1) in vivo LCMV-activated and in vitro interferon activated NK cells were more adherant to nylon wool columns than endogenous NK cells;

2) activated NK cells showed more adherance to EA monolayers than did endogenous NK cells. Since streptococcal protein A could block this adherance, this indicated the activated NK cells showed greater amounts of Fc receptor mediated adherance; 3) the LCMV activated NK cells which passed through nylon-wool columns expressed low EA adherance properties; 4) the sensitivity of NK cells to anti-θ serum plus complement increased in spleen cells 2-3 days after infection; and 5) finally, LCMV activated spleen cells were shown to contain a population of large NK cells not present in the endogenous spleen cell population. All these changes from non-activated NK cells may possibly represent an increased lytic ability of the activated NK cell. The sensitivity of spleen cells to anti-θ serum 6-7 days after activation fits well with data by Chun et al., (46) who showed that two serologically distinct populations of NK cells can be distinguished in murine spleens based on requirements for their generation and their time of appearance. In the presence of tumor necrosis serum (TNS), normal mouse spleen cells generated two peaks of natural killer cell cytotoxicity. One peak between day one and two could be abrogated by treatment with a monoclonal antibody to the Qa 5 cell surface antigen, plus complement. Qa 5 is controled by the Q region of chromosome 17. This NK population does not express the Thy-1 marker. The second peak of NK activity which occurs between day four and five is seen with or without treatment with TNS. It cannot be eliminated by anti-Qa 5 plus complement treatment and, therefore, does not express Qa 5 on its surface. The failure of BALB/C nu spleen cells to generate the second NK peak indicated to Chun et al. that T-cells were required for its generation. They tested this hypothesis by treating C57BL/6 spleen cells with anti-Thy-1.2 plus complement and found that upon addition of TNS, spleen cells would generate an early NK peak, but no late NK peak. One possibility would be that the first peak of NK activity is caused by the recruitment of pre-NK

cells stimulated by TNS. The second peak of activity is due to mature NK cells which display the Thy-1 antigen on their surface.

Other antigens associated with murine NK cell populations have been identified. Young et al., (47) and Kasai et al., (48) both found that antiserum directed against the glycosphingolipid asialo GM_1 was capable of eliminating NK activity in mice. Since incubation with antiserum alone was unable to block NK activity, apparently the structures on the cell surface which were recognized by the anitserum were not involved in the cells' lytic mechanism. Schwarting et al., (49) showed that asialo GM_1 increased in concentration in the spleen cell population with age. It reached a peak at 5-10 weeks of age, at a concentration 10-20 times that of thymocytes of neonatal splenic T-cells. This supported its role as a true differentiation antigen in the mouse. In C57BL/6 bg/bg (beige mice) which lack NK function, levels of asialo GM_1 in the splenic T-cell population do not increase with age and remain at the level of 2-3 week old normal mice.

Mouse NK cells also express the Ly5 gene product (50). Ly5 is coded for by a gene on chromosome 1 of the mouse and expressed as one of two alternative alleles in all inbred mouse strains studied to date. Treating mice with either anti Ly5.1 or 5.2 and complement eliminates NK activity. Ly5 itself is not involved in the lytic process.

In 1977 Koide et al., (51) sought to demonstrate the presence of B cell antigens on the effectors of natural cell mediated cytotoxicity in humans. Antisera with B cell specificity was produced by immunization of rabbits with papain digests of the cell membrane of spleen cells from patients with advanced histiocytic lymphoma. This antisera was found to react with normal B lymphocytes, cultured B cell lines and 70% of acute lymphocytic and acute myelocytic leukemias, but not with normal or cultured T lymphocytes. When an effector suspension of null cells isolated from peripheral blood of healthy human donors was

treated with antisera, natural cell mediated cytotoxicity was inhibited. It is left to speculation whether the B cell antisera was directed against Ir antigen or some other determinant on the cell membrane. Also, inclusion of cross reactive antibodies in the antiserum was not ruled out.

NK Versus ADCC: Comparison of Effector Populations

The question of whether natural killing and antibody dependent cellular cytotoxicity are mediated by the same or different cellular populations has not been answered definitively. Several investigators have found evidence suggesting similarities, if not common identity between the two populations. Using monoclonal antibodies to three different T-cell differentiation antigens, Fast *et al.* confirmed the findings of others that both NK and ADCC effectors reside within the population of cells rosetting with sheep erythrocytes (52). A genetic disorder corresponding to the homozygous beige gene which causes an NK deficiency in mice has been found in humans. Patients carrying the autosomal recessive Chediak-Higashi (CH) gene are defective in their ability to spontaneously lyse various tumor target cells *in vitro* by either NK or ADCC methods while other cell-mediated cytolytic functions operate normally (53).

Depletion of NK cells on monolayers of K562 tumor cells also reduced levels of ADCC. However, in most cases the NK activity was depleted to a significantly greater extent than ADCC (54). This could be explained if the NK cell, while coming from the same cell population as the ADCC effector, was more heterogeneous with regard to target recognition sites and, therefore, was adsorbed more often than ADCC effectors. In other adsorption studies lymphocytes nonadherant to target monolayers were also shown to be depleted in NK and ADCC (55). In addition, however, adherant lymphocytes showed partial depletion of NK activity when assayed immediately after separation while high levels of ADCC were still

obtained from this fraction. This would indicate different receptors and/or mechanisms involved in NK and ADCC. It was not determined whether or not both types of receptors are located on the same cell.

Using proteolytic enzymes to selectively degrade cell membrane components, Perussia et al. found that trypsin (which has no effect on Fc receptors) selectively abolished NK, but not ADCC activity (56). Treatment with pronase destroyed the Fc receptor and eliminated both NK and ADCC. Incubation for 48 hours at 37°C restored both activities. This result suggests that two distinct mechanisms, possibly both mediated by the same Fc receptor bearing cell could be responsible for NK and ADCC cytotoxicity.

Modulators of NK Activity

NK activity can be modulated by several means. One of the most widely investigated modulators of natural killing is interferon. Interferon and interferon inducers have an enhancing effect on NK levels in a four hour chromium release assay (57,58). It has been suggested that only tumor cells able to induce the generation of interferon by an effector population are susceptible to spontaneous lysis (35). Genetic differences between NK levels among individuals may be partly attributable to levels of interferon induced in vivo. In fact, correlation between resistance to infection by some diseases and the level of early NK augmentation has been observed (59). The role of interferon in this early augmentation has special significance. Ortaldo et al. showed an almost complete inhibition of NK activity due to prolonged incubation of effector cells after blockage of DNA synthesis with X-ray or mitomycin C. This activity could be partially or totally restored after a one hour incubation with interferon at 37°C (60). This indicates that interferon boosting of NK occurs independently of DNA synthesis or cell proliferation. Blockage of RNA synthesis by cyclohexamide did prevent interferon

from boosting NK activity. This suggests a dependency on protein synthesis for the ultimate boosting effect induced by interferon (61). Current information also appears to negate previous thought which had interferon producing its boosting of NK by causing a proliferation of NK effectors through cell division. A more likely role for interferon's effects would involve acting on a pool of pre-NK cells or increasing the activity of low responsive NK cells in a time and concentration dependent manner (62).

Both macrophage and NK cells have been reported to produce interferon involved in the enhancement of spontaneous target cell lysis (63,64). This supports the observation of certain tumor cell lines being more susceptible to lysis based on their ability to cause NK cells to produce interferon. Interferon boosted NK activity appears confined to large granular lymphocytes (65).

Not only does interferon augment NK activity, but according to three separate reports it also is responsible for resistance of target cells pretreated with interferon to lysis by NK cells (66-68). This interferon induced protection of target cells does not extend to killing by ADCC, thereby adding to evidence supporting non-identity between NK and ADCC mediated lysis mechanisms. Normal fibroblasts preincubated in interferon are almost totally insensitive to lysis by NK, whereas tumor cell lines or virus infected cell lines are not protected by incubation with interferon. This phenomenon does not appear to be due to inhibition of target cell binding by effector cells as seen by examination of numbers of target cell-effector cell rosettes. It is also interesting that this apparent protection of normal cells by interferon is dependent on RNA and protein synthesis, which is also characteristic of interferon in its NK augmentative role.

Prostaglandins have also been demonstrated to have NK modulating abilities. It is thought that prostaglandins exert their effect via altering intracellular calcium and cAMP levels, thus affecting membrane fluidity. One of the most

recent reports on prostaglandins shows prostaglandin E_2 (PGE_2) to actually have a dose related biphasic effect (68). Depending on the concentration and temporal factors *in vitro* PGE_2 can either augment or inhibit NK mediated cytotoxicity of target cells. This alteration in killing is not due to a change in the number of effector cells binding to targets as seen in single cell cytotoxicity assays, but is attributed to an increase in number of NK cells by recruitment of pre-NK or low cytotoxic NK subpopulations. As is the case for most immune cells, complex interactions among NK modulators effect the level of spontaneous cytotoxicity. An example of this is seen in the combined effects of prostaglandins and interferon on NK levels (69). Depending on the time span and concentrations of these two modulators either augmentated or antagonistic effects on target killing are observed. In experiments by Targan using a single cell cytotoxicity assay developed by Bonavida *et al.* (70), interferon was responsible for recruitment of additional NK subpopulations against a target cell. When followed by the addition of PGE_2 after a sufficient time span for the interferon to exert its effects, PGE_2 increased the recycling capacity of NK effectors. This increased recycling was seen by stopping a ^{51}Cr release assay at various points in time to check for the extent of target cell death indicated by supernatant levels of ^{51}Cr. Considering the complications introduced when relating this *in vitro* system to an *in vivo* system where levels of various modulators can drastically change throughout the immune system, it is obvious that much more must be known about the interaction of these immune modulators before any knowledgable manipulation of animal subjects can be achieved.

Histocompatibility Genes and Their Function

The HLA system (human lymphocyte antigen) developed out of the search for blood groups of leukocytes that could form the basis for matching donors and

recipients for bone marrow transplantation. Early work with transplantable tumors in the murine system established that genetic differences between recipient and donor tissue determined the outcome of a graft. These differences, if recognized by the immune system, lead to graft rejection. P.A. Gorer's work lead to the discovery of the mouse major histocompatibility system, H-2. The development of this system depended on the use of inbred strains of mice. The antigens relevant for transplantation matching were called histocompatibility antigens.

The human histocompatibility determinants, the HL-A antigens, are cell surface markers present on lymphocytes and most other nucleated cells. It has been found that HL-A antigens released from the cell surface by means of papain digestion are composed of two polypeptide chains. The smaller of the two glycoproteins appears invariant to the antigenic specificity carried by the larger HL-A chain. This light chain, β_2 microglobulin, has a molecular weight of 12,000 and is coded for by a gene on chromosome 15. The heavy chain has a molecular weight of 43,000 and bears the antigenic determinants that confer HLA specificity on the molecule (71). This specificity is the product of the HLA-A,-B and -C loci located on the submetacentric chromosome six (72). Histocompatibility antigens appear to be regularly dispersed over the cell membrane and separated by large empty areas.

HLA-D or D-related (DR) antigens appear to correspond or be closely linked to genes corresponding to the immune response (Ir) genes found in the murine system. It is the D(DR) locus in humans which is the primary stimulatory locus for the generation of cytotoxic effector cells in a mixed lymphocyte culture (MLC). The HLA-A, -B and -C antigens serve as the primary targets which are recognized by effector T-cells in cell mediated lympholysis, although it has been shown that antigens other than HLA-A,-B and -C can serve as target determinants (73). In addition to controlling the stimulation in mixed lymphocyte reactions the HLA-D

region has been attributed with controlling susceptibility to certain diseases and immune responses to antigens (74). The HLA-DR region is closely linked or identical to HLA-D and controls expression of a noncovalent complex of two membrane glycoproteins of apparent molecular weight 34,000 and 29,000 (75). The HLA-D (DR) region of human chromosome six has not been easily dissected due to the small number of genetic recombinant individuals studied (76). However, it is assumed from the data accumulated from other species that the HLA-D (DR) region contains multiple loci controlling the expression of different cell membrane products which are responsible for the different immune response events.

Levels of spontaneous cytotoxicity vary between individuals, suggesting that genetic differences do exert influence over natural killing. Although no direct involvement of HLA antigens affecting NK levels has been found, Santoli et al. (77), noted that effector cells from male donors carrying HLA-A3 and B7 displayed a significantly lower reactivity in spontaneous cytotoxicity when compared with lymphocytes from male donors bearing any other HLA haplotype.

Considering the control over immune responses the DR products exert, one might expect their involvement in some part of the natural cytotoxicity phenomenon. Unfortunately, while DR antigens are known to be the primary stimulus in mixed lymphocyte reactions, causing cell proliferation leading to a mature effector population, no similar developmental path can be observed in the NK subpopulation of cells. DR antigens appear on some subpopulations of MLC-stimulated T-cells (78,79). Using heteroantisera, Reinherz et al. was able to demonstrate that the population of stimulated T-cells exhibiting DR(Ia) anitgens were those with functional suppresor activity (80). Reinherz also showed that the cytotoxic T-effector cells generated in MLC did not exhibit any DR(Ia) on their membrane surface.

Histocompatibility antigen expression has also been enhanced by incubation of human peripheral blood lymphocytes in human leukocyte interferon. The appearance of HLA-A, -B and -β_2 microglobulin, but not HLA-DR(Ia) antigen was increased from 2-8 fold as determined by adsorption studies (81). David et al. were able to induce Ia expression by greater than 60% of mouse thymocyte populations stimulated with CON A or PWM (82). Indiveri et al. found similar results in nylon-wool purified T-lymphocytes from human peripheral blood (83). After PHA stimulation about 70% of both Fc_γ and Fc_μ receptor bearing T-lymphocytes exhibit Ia antigens. This is in contrast to less than 1% Ia positive cells before PHA stimulation. During the increase in Ia positive T-cells, no change occurred in the percentage of Fc_γ receptor T-cells. It remains to be determined if regulation over levels of NK can be controlled by alteration in the amounts of histocompatibility antigens expressed on cell membranes.

MATERIALS AND METHODS

Cell Lines and Culture Media

The K562 cell line used as a target in ^{51}Cr release assays was generously provided by the Salk Institute Cell Distribution Center. This cell line was derived from a patient with chronic myelogenous leukemia in blast crisis (84). The cell line was maintained in a modified Dulbecco's medium with 10% FCS.

RPMI 1640 (M.A. Bioproducts, Walkersville, MD) was the media used in all experiments and cell isolations. This media was purchased with L-glutamine already added. In addition, the RPMI was supplemented with Hepes, sodium bicarbonate and gentamicin sulfate.

Isolation of Cell Populations

Lymphocytes were isolated using the method of Boyum (85). Briefly, human mononuclear cells were isolated from the heparinized peripheral blood of healthy donors. Blood was diluted 1:4 with PBS, layered on Ficoll-hypaque (R.I. 1.3570) in 16 x 100 mm glass tubes and centrifuged for 12 minutes at 600 x g in an IEC model HN-S benchtop centrifuge with swinging buckets. The mononuclear cell layer was harvested and washed twice in PBS before further treatment.

Nylon Wool Fractionation of Lymphocytes

T-lymphocytes and null cells were obtained from isolated mononuclear cells using the nylon fiber technique of Greaves et al., (86). Briefly, scrubbed nylon

fiber (obtained from Fenwal Laboratories, Deerfield, IL) was washed in 0.2N HCL for 2 hours, rinsed in distilled water and allowed to dry. 300 or 600 mg of the fiber were submerged in saline to expel air bubbles, then packed into a 5 ml disposable syringe up to the 2.5 ml or 5 ml level. The syringe was rinsed with 50 ml of RPMI containing 10% fetal calf serum, then incubated for 30 minutes at 37°C. 5×10^7 - 1×10^8 cells in 1 - 2ml of RPMI +10% FCS were added to the column and incubated at 37°C in a 5% CO_2, 95% air atmosphere for 1 hour. Nonadherant cells were eluted with 20 ml of warmed RPMI + 10% FCS at a rate of 1 drop/second. The cells obtained were washed 3 times in RPMI + 10% FCS.

Isolation of B Lymphocytes for DR Typing

B-lymphocytes were isolated using the nylon-wool column described above. After incubating the mononuclear cell preparation for 1 hour with warm RPMI + 10% FCS, the column was packed in ice and incubated for 40 minutes. Adherant cells were then collected by washing the column with 20 ml of cold RPMI supplemented with 10% FCS at a rate of 1 drop/second. Cells obtained from this elution were washed 3 times in RPMI + 10% FCS.

T-Lymphocyte Rosetting

Preparing SRBC:

T-lymphocytes were quantitated by rosetting with sheep erythrocytes. Sheep erythrocytes (SRBC) were prepared by washing 3 times in physiologic saline. One volume of packed SRBC was incubated with 4 volumes of .143 M AET (2-aminoethylisothiouronuim bromide hydrobromide) in a 37°C water bath for 15 minutes, mixing every 5 minutes. Cells were then washed 3 times in cold saline and adjusted to a 0.1% solution of SRBC in saline.

Forming Rosettes:

Lymphocytes for rosetting were adjusted into 0.2ml volumes of 5×10^6 cells/ml. 0.8ml of AET treated SRBC were incubated with 0.2ml of lymphocytes for 15 minutes in a 37°C water bath. After this incubation, cells were pelleted and incubated on ice for at least 1 hour. After incubation cells were gently resuspended and rosettes were counted. Any lymphocyte with at least 3 erythrocytes attached was considered a rosette.

EAC Rosettes

Preparing SRBC:

SRBC were washed 3 times in saline. 9.5 ml of hemolysin diluted 1:500 was added to 0.5 ml of washed SRBC and incubated for 30 minutes at 37°C. After incubation the cells were washed 3 times in cold saline. After the final wash the supernatant was discarded and the pellet resuspended in 10 ml of fresh human serum diluted 1:20 with saline. The preparation was incubated for 30 minutes at 37°C then centrifuged at 300 x g and the pellet resuspended in saline to make a 1.0% suspension.

Forming Rosettes:

0.25 ml of a 5×10^6 cells/ml suspension of lymphocytes in saline were added to 0.25 ml of SRBC-EAC. This mixture was centrifuged for 2 minutes at 200 x g and incubated for 30 minutes at room temperature. After incubation the mixture was vortexed for 15 seconds and 2/3 of the supernatant discarded. Any lymphocyte with 3 or more bound SRBC was considered a rosette.

HLA-A-B, and C Locus Typing

The 2-stage microlymphocytotoxicity test was performed according to NIH guidelines. Briefly, 1 µl of cells (cell concentration 2 x 10^6/ml) to be tested was dispensed into oiled microtiter trays containing antiserum to HLA-A, B and C loci and allowed to incubate at room temperature for 45 minutes. 5 µl of rabbit complement was then added, followed by 90 minutes of incubation at room temperature. After this incubation, 5 µl of eosin was added, followed 5 minutes later by 5 µl of formalin. A coverglass was then applied and the edges sealed with paraffin.

HLA-DR Typing

Antiserum for typing the DR locus was generously provided by Dr. Renee Duquesnoy of the Milwaukee Blood Center. DR typing of cells was performed according to the method of Paul Terasaki at UCLA. Briefly, 1 µl of cells (cells adjusted to 2 x 10^6/ml) and 1 µl of antisera were incubated for 1 hour at 37°C on an oiled microtiter tray; incubated with 5 µl rabbit complement for 2 hours at room temperature; then incubated with eosin for 5 minutes. Cells were fixed with formalin and trays sealed with a coverglass and paraffin. The cells were allowed to settle for at least 2 hours before the trays were read using an inverted microscope.

Treatment of Cells Used in ^{51}Cr Release Assay

DR Antisera Treated Cells:

Cells to be treated with DR antisera were incubated in 0.33 ml of RPMI + 10% FCS and 0.33 ml of DR antiserum at an appropriate dilution and placed in a 37°C incubator for 1 hour. After 1 hour 0.33 ml of undiluted rabbit complement was added and this mixture was incubated for 2 hours at room temperature. After

incubation with complement, cells were washed 3 times in RPMI + 10% FCS and used in the ^{51}Cr release assay. Cells treated with DR antisera only were incubated with the appropriate concentration of antisera for 1 hour at 37°C. Cells treated with complement only were incubated with rabbit complement for 2 hours at room temperature.

Stimulation of Cells Used in ^{51}Cr Release Assay

Cells to be stimulated with IFN or PHA were isolated one day before use in the ^{51}Cr release assay. 5×10^6 cells in 1 ml of RPMI + 10% FCS were incubated for 18 hours with either 1000 U IFN supplied by the Michigan Department of Public Health or a dilution of phytohaemagglutinin (Welcome Reagents Limited Beckenham, England) in RPMI sufficient to provide a final PHA concentration of 1:40. After 18 hours the cells were washed 3 times in RPMI + 10% FCS and adjusted to appropriate concentrations for use in ^{51}Cr release assays.

^{51}Cr Release Assay for NK Cytotoxicity

A 4 hour ^{51}Cr release assay against K562 target cells in microtiter tray wells was performed to determine target cell lysis. $5\text{-}7 \times 10^6$ K562 cells were incubated with 100 µCi of ^{51}Cr for 1 hour in a 37°C water bath. After 1 hour cells were pelleted and washed 3 times in RPMI + 10% fetal calf serum and placed in wells of a Linbro 96 round bottom well tray (Hamden, CT). Effector lymphocytes were added in 0.1 ml of RPMI + 10% FCS in appropriate ratios for each experiment. All effector:target ratios including those for determining the maximum and spontaneous release levels were performed in triplicate. Maximum ^{51}Cr release values were obtained by incubating 10^4 ^{51}Cr labeled K562 cells with 0.1 ml of a 2% solution of Triton X-100 detergent (Research Products International Corp., Elk

Grove Village, IL). Spontaneous release values were obtained by incubating 10^4 ^{51}Cr labeled K562 cells in 0.2 ml RPMI + 10% FCS for 4 hours. Trays were centrifuged for 3 minutes at 50 x g in an International Equipment Company model PR2 refrigerated centrifuge to pellet the cells and allow more surface contact between effectors and targets. Trays were incubated for 4 hours in a 37°C incubator with a 95% air, 5% CO_2 environment. After incubation, supernatants were harvested using the Titertek Supernatant Collection System (Flow Laboratories, Norway) and radioactivity determined in a gamma counter.

RESULTS

NK Activity of Nylon-Wool Passed Lymphocytes Treated with DR Antiserum

To determine whether the population of cells responsible for NK activity expressed HLA-DR antigens on their surface, cells were treated with HLA-DR antisera directed against the allotypic determinants of the DR molecule. The subjects were typed for their HLA-A, B, C and DR locus specificities. The highest dilutions of antisera and complement which allowed for maximum killing of lymphocytes in this system were used. Nylon-wool nonadherant cells were used in all NK assays. The nylon-wool separation procedure used gave nonadherant cell populations with no less than 92% AET-SRBC rosetting cells and no greater than 8% EAC rosetting cells. In experiments on 3 subjects, untreated control cells were compared to cells treated with appropriate and inappropriate DR antiserum as well as antiserum directed against the subject's HLA-A or B loci. This served as an additional control since it is already known that NK cells possess HLA-A, -B and -C determinants on their surface. NK assay results were grouped into 2 ranges of effector:target ratios. Both of the ranges used were found to be on the linear part of the cytotoxicity curve. Using a test of least significant difference, no significant difference at the 5% level could be found in NK levels between cells treated with the appropriate DR antiserum plus complement and the untreated control cells (Table 1). This was true for both groups of effector:target ratios. Significant difference at the 5% level could be found, however, between cells treated with the appropriate HLA-A or B locus antiserum plus complement and the untreated controls in all 3 subjects. This was true for both groups of effector:

TABLE 1

SUBJECT: J.H.	% CYTOTOXICITY HIGH RATIO	LOW RATIO	SUBJECT: M.O.	% CYTOTOXICITY HIGH RATIO	LOW RATIO	SUBJECT: R.B.	% CYTOTOXICITY HIGH RATIO	LOW RATIO
TREATMENT	35:1	17.5:1	TREATMENT	45:1	22.5:1	TREATMENT	45:1	11:1
Control Untreated	43	31	Control Untreated	80	69	Control Untreated	72	64
Undiluted C'	45	27	Undiluted C'	87	68	Undiluted C'	81	66
DR7 antiserum plus C'	42	24	DR5 antiserum plus C'	77	59	DR2 antiserum plus C'	78	52
DR5 antiserum plus C'	48	28	DR2 antiserum plus C'	72	61	MT3 antiserum plus C'	81	57
DR7 antiserum	37	30	DR5 antiserum	72	64	DR2 antiserum	80	54
DR5 antiserum	48	33	DR2 antiserum	73	52	MT3 antiserum	83	62
HLA-B35 antiserum plus C'	5	5	HLA-A2 antiserum plus C'	34	20	HLA-A2 antiserum plus C'	20	12

TABLE 1 (continued)

SUBJECT: J.H.	% CYTOTOXICITY HIGH RATIO	LOW RATIO
TREATMENT	50:1	12.5:1
Control Untreated	47	22
Undiluted C'	60	25
DR7 antiserum plus C'	61	24
DR5 antiserum plus C'	70	31
DR7 antiserum	62	30
DR5 antiserum	67	29
HLA-B35 antiserum plus C'	15	8

4 hour ^{51}Cr release assay
Experiments had 13 and 10% spontaneous release levels respectively

J.H. = HLA-B35, DR(5,-)

SUBJECT: M.O.	% CYTOTOXICITY HIGH RATIO	LOW RATIO
TREATMENT	56:1	14:1
Control Untreated	76	47
Undiluted C'	72	38
DR5 antiserum plus C'	76	42
DR2 antiserum plus C'	73	36
DR5 antiserum	71	46
DR2 antiserum	73	38
HLA-A2 antiserum plus C'	35	14

4 hour ^{51}Cr release assay
Experiments had 9 and 13% spontaneous release levels respectively

M.O. = HLA-A2, DR(2,7)

SUBJECT: R.B.	% CYTOTOXICITY HIGH RATIO	LOW RATIO
TREATMENT	40:1	10:1
Control Untreated	62	31
Undiluted C'	68	37
DR2 antiserum plus C'	58	39
MT3 antiserum plus C'	72	48
DR2 antiserum	60	31
MT3 antiserum	74	49
HLA-A2 antiserum plus C'	25	15

4 hour ^{51}Cr release assay
Experiments had 21 and 14% spontaneous release levels respectively

R.B. = HLA-A2, DR(4,5)

MT3 antiserum has anti-DR 4,7,9 activity.

target ratios. NK levels for cells treated with the appropriate HLA-A or B locus antisera and no complement were not found to be reduced (data not shown).

NK Activity of Nylon-Wool Passed Lymphocytes Stimulated with Interferon or PHA

Nylon-wool passed PBL were stimulated with either interferon or phytohaemagglutinin in an attempt to alter the antigenic profile of their membranes and induce the display of HLA-DR determinants. NK assays were performed with nylon-wool separated cell populations after 18 hour incubations with 1000 U of type I interferon or PHA (Wellcome) diluted to a 1:40 final concentration. After the 18 hour incubation period, cells were treated with appropriate and inappropriate HLA-DR antiserum plus complement. A control was also included in which cells were incubated with the appropriate HLA-A or B antiserum plus complement. After these treatments, cells were then used in 4 hour NK assays. These NK assays were performed at low effector:target ratios in order to observe even small effects of the stimulators on target cell lysis levels. Significant differences at the 5% level were found between the control and treatments involving PHA stimulated cells for subject J.H. (Table 2). Differences were also found between control untreated cells and PHA stimulated cells treated with serum directed against the subject's HLA-A or B locus plus complement (subject R.B.). Differences between control cells and PHA stimulated cells for subject M.O. seem apparent, but due to lack of sufficient experimental repetition no statistical significance can be drawn.

TABLE 2

SUBJECT: J.H.	% CYTOTOXICITY	SUBJECT: M.O.	% CYTOTOXICITY	SUBJECT: R.B.	% CYTOTOXICITY
TREATMENT	6:1 RATIO	TREATMENT	6:1 RATIO	TREATMENT	6:1 RATIO
Control Untreated	19,19	Control Untreated	28,N.A.	Control Untreated	43,75
1000 U IFN	14,22	1000 U IFN	27,N.A.	1000 U IFN	39,62
PHA @ 1:40 dil.	66,40	PHA @ 1:40 dil.	48,N.A.	PHA @ 1:40 dil.	66,74
1000 U IFN and DR7 antiserum + C'	8,11	1000 U IFN and DR5 antiserum + C'	18,N.A.	1000 U IFN and DR7 antiserum + C'	33,52
1000 U IFN and DR5 antiserum + C'	12, 7	1000 U IFN and DR2 antiserum + C'	19,N.A.	1000 U IFN and MB3 antiserum + C'	27,45
PHA @ 1:40 dil. and DR7 antiserum + C'	37,42	PHA @ 1:40 dil. and DR5 antiserum + C'	39,N.A.	PHA @ 1:40 dil. and DR7 antiserum + C'	62,75
PHA @ 1:40 dil. and DR5 antiserum + C'	38,31	PHA @ 1:40 dil. and DR2 antiserum + C'	40,N.A.	PHA @ 1:40 dil. and MB3 antiserum + C'	70,52
Undiluted C'	12,10	Undiluted C'	31,N.A.	Undiluted C'	25,62
1000 U IFN and HLA-B35 antiserum + C'	10, 7	1000 U IFN and HLA-A2 antiserum + C'	5,N.A.	1000 U IFN and HLA-A2 antiserum + C'	13,15
PHA @ 1:40 dil. and HLA-B35 antiserum + C'	39,23	PHA @ 1:40 dil. and HLA-A2 antiserum + C'	12,N.A.	PHA @ 1:40 dil. and HLA-A2 antiserum + C'	38,56

4 hour ^{51}Cr release assay
Experiments had 7 and 8% spontaneous release levels respectively.

J.H. = HLA-B35, DR(5,-)

4 hour ^{51}Cr release assay
Experiment had 7% spontaneous release level.
N.A. = Not Available

M.O. = HLA-A2, DR(2,7)

4 hour ^{51}Cr release assay
Experiments had 17 and 12% spontaneous release levels respectively.

R.B. = HLA-A2, DR(4,5)
MB3 antiserum has anti-DR 4,5 activity.

DISCUSSION

A previous study by Ng et al., (95) using a monoclonal antiserum directed against common structures of HLA-DR antigens suggested that the cell population responsible for NK activity was devoid of HLA-DR antigens on its surface. This was in contrast to earlier reports (96,97) that used specific xenoantisera to characterize NK effectors as B lymphocytes. This study has confirmed the results of Ng et al., by using antiserum derived from humans against the allospecific structures of the DR molecule. The DR antisera plus complement used to treat nylon-wool non-adherant PBL populations was unable in every subject to reduce levels of NK cell mediated cytotoxicity.

Previous findings (98-101) indicate that NK cells display some T-cell characteristics and that NK activity is recovered in T-cell populations. It has also been observed that T-cells can display HLA-DR antigenic determinants under appropriate conditions. This study showed that anti-DR sera screened for its high degree of reaction with the allospecific structure of DR antigens was unable to eliminate the NK function of a nylon-wool passed PBL population against the K562 target cell.

In this study the cell population(s) responsible for NK activity were stimulated in an attempt to cause the appearance of DR antigen. Interferon exerts many effects at the surface of the cell membrane. It is well documented in its ability to boost levels of natural killing against the K562 target cell (102-104). Using type I interferon supplied by the Michigan Department of Public Health, no increased levels of NK over untreated controls were observed. Furthermore, no decrease in NK activity indicative of the appearance of DR antigen on the cell

surface was observed after treatment with anti-DR serum plus complement. The lack of boosting of NK levels by the interferon leaves open the possibility that the cell population was not, in fact, stimulated. This aspect will be addressed in additional experiments using interferon from a different source.

Treatment of nylon wool passed PBL with PHA produced interesting results. Statistically significant boosting of cytotoxicity against the K562 target occurred in subject J.H. (Table 2, treatment 3) and also apparently in subject M.L., (Table 2, treatment 3) although only one experimental trial was performed for this subject. This phenomenon of mitogen induced cytotoxicity against NK targets has been frequently observed (105-110) and is referred to as activated lymphocyte killing (ALK). This type of killing is thought to be a property of activated T-cells and not due to the survival of NK cells, whose presence decrease over several days in culture. Studies that have examined ALK by PHA stimulated blasts (105) have usually stimulated lymphocytes with PHA for at least 3 days. The present study shows that in subjects J.H. and M.O. an 18 hour incubation of nylon-wool nonadherant PBL with PHA was sufficient to allow the detection of this form of killing. A high cytotoxic level in the untreated control for subject R.B. prevents the difference between PHA stimulated lymphocytes and control cytotoxicity levels from being statistically significant. In all 3 subjects, stimulation of the effector cell population(s) with interferon or PHA does not cause the expression of HLA-DR on the surface of the NK cell, as seen by the inability of treatment with appropriate anti-DR serum plus complement to abrogate killing. Treatment of interferon stimulated cells with the appropriate HLA antiserum plus complement decreased cytotoxicity in all subjects. An interesting finding was the inability to reduce cytotoxicity levels in PHA stimulated PBL to the levels of cells stimulated with interferon after treatment with appropriate HLA antiserum plus complement. The sustained high cytotoxicity levels in all 3 subjects suggest 2 possible explana-

tions. Either the cells responsible for the target killing do not express HLA - A,B,C antigens on their membrane surface or the cell population(s) responsible for cytotoxicity were still present in large enough numbers after treatment with the antiserum plus complement to cause the high level of target lysis seen. Since effector cell concentrations were adjusted immediately before the assay, an increased number of effector cells in the tray wells seems doubtful.

Recent findings by Timonen et al., (111) suggest that considerable antigenic shift at the NK cell membrane surface does occur upon long term culture of this cell population. It was reported that expression of Ia (DR) antigens detected by monoclonal antibodies dramatically increases on the NK cell surface upon culture. This information lends support to the idea of NK cells being T-cell precursors. However, in maintaining cells under long term culture conditions, medium containing supernatants of stimulated T-cells is required. Due to the phenomenon of ALK, which appears to be less specific in its target cell restrictions, caution must be maintained in not confusing the cell populations responsible for natural cytotoxicity and activated lymphocyte killing. Further investigation of ALK effector populations should be undertaken in order to more clearly define the distinctions between this and other cytotoxic effector cells grown in long term culture.

APPENDIX

APPENDIX

EXPERIMENT NUMBER ONE

In order to make certain that removing B-cells and other nylon-wool adherant cell populations from peripheral blood lymphocytes did not diminish natural killing levels, a comparison was made between NK levels obtained from PBL which were and were not passed through nylon-wool columns.

MATERIALS AND METHODS

Isolation of cell populations and nylon-wool fractionation of lymphocytes was performed as described elsewhere. Natural killer cell assays using ^{51}Cr release as a measure of target cell lysis was performed as described elsewhere. K562 target cells were generously provided by the Salk Institute Cell Distribution Center.

RESULTS AND DISCUSSION

No significant difference at the 5% level could be found between NK cytotoxicity levels from fractionated and unfractionated populations using a test of least significant difference (Table 3). Statistical testing was only performed on data from subjects used in 2 or more experimental runs. These results agree with previous data (112) showing no decrease and sometimes a slight increase in NK levels from nylon-wool purified peripheral blood lymphocytes.

Spontaneous Release was 8% or less for all experiments

TABLE 3

	SUBJECT: J.H.		SUBJECT: D.T.	
RATIO	Whole PBL Pop.	Nylon-Wool Nonadherant PBL	Whole PBL Pop.	Nylon-Wool Nonadherant PBL
100:1	72,75	74,74	79,73	73,80
50:1	68,71,70	54,72,64	64,73,70	63,76,65
25:1	49,66,64	43,59,62	48,67,65	56,77,64
12.5:1	30,50,44	25,42,52	34,63,53	34,62,52
6:1	22	30	40	31
3:1	12	16	21	20

Spontaneous Release = 10%

	SUBJECT: R.B.		SUBJECT: R.A.	
RATIO	Whole PBL Pop.	Nylon-Wool Nonadherant PBL	Whole PBL Pop.	Nylon-Wool Nonadherant PBL
50:1	73,68	72,70	88	69
25:1	69,63	73,60	65	70
12.5:1	57,55	63,58	52	59
6:1	49,51	56,48	30	42
3:1	31,28	36,31	18	28

Spontaneous Release = 8%

	SUBJECT: D.K.		SUBJECT: M.O.	
RATIO	Whole PBL Pop.	Nylon-Wool Nonadherant PBL	Whole PBL Pop.	Nylon-Wool Nonadherant PBL
50:1	35	38	57,62	53,65
25:1	24	27	56,51	42,59
12.5:1	15	17	45,44	23,55
6:1	7	10	26,32	11,37
3:1	4	5	12,18	7,25

Spontaneous Release = 6%

	SUBJECT: R.G.		SUBJECT: T.M.	
RATIO	Whole PBL Pop.	Nylon-Wool Nonadherant PBL	Whole PBL Pop.	Nylon-Wool Nonadherant PBL
50:1	80	72	70	54
25:1	59	55	61	43
12.5:1	40	36	41	28
6:1	19	21	21	14
3:1	10	11	11	7

EXPERIMENT NUMBER TWO

An estimate of the percentage of nylon-wool passed PBL binding to K562 targets was obtained using the K562 monolayer adsorption technique of Silva et al., (113). This assay allowed a rough approximation of the maximum number of nylon-wool nonadherant PBL which could be involved in the natural cytotoxicity process.

MATERIALS AND METHODS

Preparation of CRBC monolayer plates: 1 ml of poly-L-lysine (50 µg/ml in PBS) was added to each 35 x 10 mm plastic tissue culture plate (Falcon Plastics, Oxnard, CA) to be prepared. Plates were incubated for 1 hour at room temperature then washed 3 times in PBS. 1 ml of a 1% solution of chicken red blood cells (CRBC stored in alsevers solution and kept less than 2 weeks) was added and plates incubated for 1 hour at room temperature. After incubation plates were washed 3 times in PBS. Plates were then incubated with 0.2% glutaraldehyde in PBS for 10 minutes followed by 3 washes in PBS. Next, plates were incubated 10 minutes in 0.1 M glycine in PBS. After this incubation plates were washed in PBS, covered with sterile PBS and stored at $4^{o}C$ until use. Plates at this stage were used as controls in all experiments.

Adhering K562 cells to CRBC plates: Plates were washed in PBS after removal from $4^{o}C$ storage. 1 ml/plate of a 1:50 PHA solution was added and plates incubated for 30 minutes at $37^{o}C$ in a 5% CO_2, 95% air incubator. After incubation plates were washed 3 times in PBS and 3×10^7 K562 cells in 1 ml PBS was added to each plate. Plates were incubated at $37^{o}C$, 5% CO_2 for 30 minutes. After incubation plates were washed 3 times and 1 ml/plate of 1% human RBC was added. Plates were incubated for 30 minutes at $37^{o}C$, 5% CO_2. After incubation plates were washed 3 times in PBS. Nylon-wool passed lympocytes to be tested for

adherance were added (5×10^6 cells in 1 ml of RPMI + 10% FCS/plate) and incubated at 37°C, 5% CO_2 for 2 hours. Following adsorption, media was removed and pooled with another 2 washes of 2 ml RPMI + 10% FCS from each plate.

RESULTS AND DISCUSSION

In each experimental trial, the number of lymphocytes eluted from K562 monolayer plates after the 2 hour incubation was compared to the number of lymphocytes eluted from control plates coated only with a monolayer of CRBC. Data from these experiments is shown in Table 4. The percentage of eluted cells was determined by the formula:

$$\frac{\text{\# of PBL eluted from K562 monolayer plates}}{\text{\# of PBL eluted from CRBC control plates}} \times 100$$

TABLE 4

Cells eluted from K562 monolayer

Subject	% cells eluted	average	average % adherant cells
S.N.	65,66,55	62	38
J.H.	54,67,55,57,69	60	40
R.B.	44,38,44	42	58
F.S.	67,56,78,67	67	33

By averaging the data obtained throughout the experiment on each subject, the number of cells adherant to the K562 monolayer after incubation falls into a range from 33 to 40% for subjects F.S., S.N. and J.H. Subject R.B. appears to have higher numbers of PBL (58%) adherant to the K562 monolayer cells. This observation seems to be supported by higher levels of cytotoxicity in NK assays in comparison to subject J.H. (Table 3). If this data is supported statistically after more experimental repetitions, it would agree with the findings of Timonen et al.,

(112) which state that absolute levels of cytotoxicity in NK assays are determined by the number of effectors binding to targets as well as the amount of effector cell recycling which occurs.

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