NATURAL CELL-MEDIATED IMMUNITY AGAINST TUMORS

Edited by Ronald B. Herberman

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PREFACE

Most immunologists have long tended to regard natural or spontaneous immunity as an amorphous subject of rather dubious import. In part, this view was due to traditional training and approaches to immunologic problems. By comparison with acquired immunity, and the extensive information on its characteristics, poorly understood observations of natural immunity met with much skepticism about their biologic significance. Furthermore, immune responses are generally considered to be induced by a particular, known antigen. There is almost an instinctive belief of immunologists that all immune phenomena must be in response to an immunogen. Thus, uninduced reactivity, not under the control of the investigator and directed against ill-defined structures, is suspect. The prevailing view has been that natural immunity differs from acquired immunity only in the nature of the inducing antigen or in some quantitative aspects, but that the mechanisms involved are the same. Efforts were often directed toward identification of an environmental or endogenous antigenic stimulus and, if none were found, the reactivity would be judged nonspecific or even nonimmunologic.

Changes in this attitude have been slow to develop and a beginning appreciation of the potential of this spontaneously arising state of immune reactivity has only recently come to the fore. As the extensive investigations on NK cells attest, this traditional view was based on a tacit assumption rather than a coherent analysis and was unwarranted, for it is now clear that in many ways the cellular basis and regulatory factors for natural immunity differ strikingly from those involved in "classical" acquired immunity. Indeed, in certain key biologic situations such as the germ-free state, and in nude or thymectomized mice, natural immunity remains quite unimpaired. Moreover, in neoplasia this noninduced cellular system may in some situations make a more effective contribution to host resistance and survival than the highly vaunted T cell-based immune cytotoxicity.

We are entering a new era in which the mechanism and operational basis for the host's innate capability to cope with threats of the environment are undergoing far-reaching reassessment. Investigators in this neglected sector of modern immunobiology now proceed apace, being

limited mostly by the need for development of requisite techniques, reagents, and approaches, a new awareness of the implications of noninduced resistance and its possible complementary role or even primary role in host defenses, and the abandonment of past bias.

Natural killer (NK) cells were discovered only about eight years ago. Thereafter, a small number of investigators became interested in this subpopulation of lymphoid cells and began to explore their characteristic ability to lyse a wide variety of tumor cells and their possible role *in vivo*. More recently, with the marked burgeoning of interest in natural cell-mediated immunity, experienced investigators worldwide have turned to this field. NK cells are now known to be present in a variety of animal species and their characteristics have been extensively studied. Although the reactivity of NK cells against tumors continues to attract most of the effort, the field has diversified in a number of important aspects: It has become evident that the reactivity of NK cells is by no means restricted to tumor cells as these cells also recognize and lyse certain normal cells, including subpopulation of thymocytes, macrophages, and bone marrow cells. The reactivity against bone marrow cells has provided a major new insight into the mechanisms that could account for natural resistance to engraftment of allogeneic and xenogeneic bone marrow. Thus, the NK cell may well be involved in regulation of differentiation of normal hematopoietic cells, as well as in resistance against tumors. Moreover, NK cells may also have a role in resistance to viral diseases as they have been found to be quite active against cells infected with a variety of viruses.

Research on NK cells has extended into other fields of investigation and has contributed new knowledge on problems in these areas. The accumulating evidence for a close relationship of NK cells to K cells [effector cells for antibody-dependent cell-mediated cytotoxicity (ADCC) against tumors] has helped in the characterization of K cells. Similarly, the discovery that interferon plays a central role in augmentation of NK activity reveals an appealing new interpretation for the antitumor effects of this agent.

Research on natural cell-mediated immunity has identified other categories of natural effector cells, particularly macrophages and granulocytes. It is especially noteworthy that other cytotoxic effector cells have also been discerned, which have a number of differences from, as well as some similarities with, NK cells. Moreover, natural cell-mediated immune functions apart from cytotoxicity are now perceived, such as the natural production of lymphokines in response to tumor cells and other stimuli.

Much of the research covered in this volume got underway at the very time major changes were taking place in the prevailing views regarding tumor immunology. Much of the deep-seated belief that immonology was

destined to play a major role in understanding and coping with cancer centered around three concepts that enjoyed wide acceptance: (a) the presence of tumor-specific or tumor-associated antigens on most or all tumors; T cell-mediated immunity against these antigens, especially cytotoxic T cells, was considered to play a central role in resistance against tumor growth; (b) immune surveillance against tumors, again with T cell-mediated immunity assigned paramount importance; (c) implicit faith that immunotherapy represented a major advance in dealing with the realities of clinical cancer. Here, too, the role of T cells, especially cytotoxic T cells, was the major focus. Considerable efforts have been made to account for the effects of immunotherapy by the induction or increase in T cell-mediated immunity or by the elimination of factors interfering with this arm of the immune response. Extensive immunological monitoring of cancer patients receiving immunotherapy focused on the levels of T cells and their functions and on specific T cell-mediated immunity against tumor-associated antigens.

Each of these components of the foundation of faith in, and enthusiasm for, tumor immunology has now been seriously challenged, and painstaking efforts are directed toward separating reality from unrealistic expectations. This questioning of the importance of the immune system in resistance against cancer results from findings that spontaneous tumors are often nonimmunogenic, the absence of specific cytotoxic T cells reactive against many human tumors (indeed, much if not most of what had been previously described is likely due to NK cells or other naturally cytotoxic cells), apparent contradictions to some major predictions of the immune surveillance hypothesis (e.g., lack of a major increase in tumor incidence in nude, athymic mice, or in neonatally thymectomized mice), and failure to demonstrate convincingly efficacy in most of the clinical trials of immunotherapy (accompanied by the frustrating failure to detect clear effects on the immunologic parameters that were monitored, even in those trials where immunotherapy was actually providing clinical benefits).

The discovery and understanding of NK cells and other natural effector cells have revealed new vistas for coping with some of these problems and have given a new perspective to the concept of immune surveillance and a distinctive role for the immune system in resistance against tumor growth, even in T cell-deficient individuals and against nonimmunogenic tumors. These developments suggest new possibilities for immunotherapy and alternative explanations for some of the beneficial effects of immunotherapeutic agents in current use.

This volume is designed to provide the first comprehensive treatment of the subject of natural cell-mediated immunity against tumors. Up to now, only selected aspects of NK cell research has been dealt with in review articles. With the rapid proliferation of information on NK and

other natural effector cells, it has become progressively more difficult for most experimentalists and clinicians to sort through and assimilate this complex literature. The nature of the NK cell and its relationship to the known categories of lymphoid and other hematopoietic cells has proved to be especially confusing. There has been a shift from the earlier consideration of the NK cell as having no distinguishing characteristics, and consequently described as a null cell, to the present where the cell has been reported to have, in fact, a variety of positive features, some suggestive of a relationship to T cells and others of a possible link to macrophages or even to B cells. With the identification of other forms of natural cell-mediated immunity, it has become increasingly difficult to determine which effects are due solely to NK cells and to other effector cells, or even to distinguish adequately between these various cell types. It therefore became essential to sift through this accumulated information and to systematize and assess the data and identify the current problems, issues, and delineate the needs, opportunities, and prospects for this research area.

This volume brings together contributions from the leading laboratories presently conducting research in this field. Each was invited to summarize one or more main aspects of their research, in a brief but relatively comprehensive fashion, and was encouraged to emphasize current information, including unpublished data. In view of the immediate character of these contributions, the editor and the publisher had committed themselves to seeing this volume to completion within six months of submission of manuscripts. The brevity of the processing period necessitated the utilization of "camera ready" format. Hopefully, the benefits of speedy publication will outweigh any inconveniences such as inconsistency in style, typeface, and typographical errors. Regrettably, a few of the important contributors to the field were unable to meet the short deadline and, hence, the omission of their contributions. However, the overall response and cooperation in this plan has been excellent and each area appears amply documented. Since the planned schedule has been realized, the overall product reflects a truly current exposition of the state of knowledge in the field.

The main topics to be covered were selected in the planning and organization of this volume. Rather than having contributors prepare a review on all aspects of their investigations on natural immunity, they were asked to segregate their information into segments relevant to the main topics that make up the structure of this volume. This made for the integration of information on each major topic, avoiding the diffuse quality that would otherwise result. To further assist the reader in evaluating and comprehending the diverse range of complex data, the editor has prepared descriptive, critical synopses for each section, highlighting the main points.

This volume seeks to provide a better perspective of how these natural mechanisms fit in with and relate to the traditional, more extensively studied components of the immune system. It is now apparent that many unexplained phenomena that have been noted during the course of immunologic research, e.g., increased baseline response or unexpectedly rapid or "nonspecific" responses to immunization, may well involve NK cells or other natural effector mechanisms. It therefore becomes mandatory to consider and control for the possible contributions of NK cells in studies of "immune" T cell reactivities. Similarly, those concerned with macrophages and granulocytes must also consider the natural activity of these effector cells. Indeed, as investigators proceed apace, it becomes compellingly evident that these natural, noninduced effectors constitute a very broad spectrum of cell types, primarily perceived by their distinct activities, their membrane characteristics, susceptibility to modulation, genetics, etc. They transcend the few neatly categorized cell types that heretofore have dominated immunobiologic investigation.

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INTRODUCTION

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The chapters in this book are mainly summaries of current information regarding natural killer (NK) cells and other aspects of natural cell-mediated immunity. To put this large amount of data into perspective, it is worthwhile to briefly review the chronology of developments in this rapidly advancing field of research.

Cell-mediated cytotoxicity against tumors first began to be extensively examined about 13 years ago, when Hellström (1967) developed the colony inhibition assay. Cytotoxicity against a variety of experimental tumors in rodents and against cultured cells from some human tumors was observed with lymphoid cells from tumor-bearing or tumor-immune individuals. Shortly thereafter, the technically simpler microcytotoxicity assay was developed (Takasugi and Klein, 1970) and with this many laboratories began studies on cell-mediated immunity against tumors. During the next two to three years, there were many reports on cell-mediated cytotoxicity against tumors in rodents and other experimental species. There was even a more rapid and extensive proliferation of reports on reactivity of cancer patients, with a wide assortment of tumor types (for review of these assays and findings, see Herberman, 1974). Both the colony inhibition and microcytotoxicity assays required the rather tedious visual counting of large numbers of cells. Shortly after Brunner *et al.* (1968) developed the ^{51}chromium release cytotoxicity assay, this and other radioisotopic release techniques began to be applied to tumor systems (Oren *et al.*, 1971; Lavrin *et al.*, 1972, 1973; Leclerc *et al.*; 1972, Jagarlamoody *et al.*, 1971; Cohen *et al.*, 1973). Particularly for studies with leukemia or lymphoma target cells, it was possible to perform ^{51}Cr release assays in 4 hours and this rapid and quantitative

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assay has become the method of choice for most studies of lymphocyte cell-mediated cytotoxicity.

During this early period of research, the focus was on specific cell-mediated cytotoxicity against tumor-associated antigens. Tumor-bearing or tumor-immune individuals were found to react against their own or related tumors but usually not against skin fibroblasts or unrelated tumors. Since normal individuals were assumed to be unreactive, the effects of normal lymphocytes were taken as the baseline control or zero point and results were expressed as the activity above that of the normal control. In studies with neuroblastoma, Hellström *et al*. (1968) noted that some normal relatives of patients reacted but suggested that this was due to some contact-induced sensitization against the disease.

Although this concept of almost ubiquitous, specific tumor associated cell-mediated cytotoxic reactivity against tumors became generally accepted, some exceptions to the expected specificity in clinical testing began to be noted by a few investigators. This led to a Conference and Workshop on Cellular Immune Reactions to Human Tumor-Associated Antigens, held in June 1972 (for proceedings and discussion, see Herberman and Gaylord, 1973). Many problems with the cytotoxicity tests with human tumor cells were apparent, but the majority of participants considered these to be due to technical difficulties with the target cells and/or with the preparation of the effector cells. However, a few reports were discussed which indicated that normal individuals, including those unrelated or unexposed to cancer patients, could react against leukemic cells (Rosenberg *et al*., 1972) or against cell lines derived from tumors (McCoy *et al*., 1973; Oldham *et al*., 1973). Because of this, the following comments were included in the concluding remarks at the Conference: "Most of us have seen a variety of effects produced by the lymphocytes of normal individuals. Does this represent real immunologic activity against tumor-associated antigens, or is this just noise or problems with setting the baseline in the assays?... It is certainly possible that some or all normal individuals have immune reactivity against tumor cells or cell lines derived from tumors...These reactions could play an important role in immune surveillance" (Herberman, 1973).

The discussion at this Conference stimulated much reevaluation of disease-related specificity by many of the participants and in fact, by the time of submission of their manuscript for the Monograph, Skurzak *et al*. (1973) included a description of reactivity by non-malignant controls against glioma target cells. Within the next 1-2 years, many investigators, including some of those initially obtaining good specificity, reported on cytotoxic reactivity by normal

individuals and a lack of complete histologic type-specific reactivity by cancer patients (Takasugi *et al.*, 1973; Heppner *et al.*, 1975; Peter *et al.*, 1975; Kay *et al.*, 1976; Canevari *et al.*, 1976).

In the midst of this transition period, in November 1974, there was a Workshop on Cell-Mediated Cytotoxicity for Bladder Carcinoma (see Bean *et al.*, 1975 for report) at the Sloan-Kettering Institute in New York. The focus was on human bladder cancer and secondarily on malignant melanoma, since these were thought to be the main types of cancer for which specific, disease-related cell-mediated cytotoxicity still could be demonstrated. From comparative tests by several groups on a limited number of blood specimens, it was clear that differences in preparation of effector cells did affect the results. However, the most impressive outcome was that, despite these differences, there was rather good correlation in results among investigators. Since at the Workshop and in most of the investigators' own laboratories, effector cells frequently showed reactivity restricted to some of the target cell lines, Dr. Eva Klein suggested the new operational term, selective cytotoxicity, to denote cytotoxicity for some target cells, including, for the cancer patients tested, those of unrelated histological types, but not for all test target cells. In retrospect, and as illustrated by some of the chapters in this book, this appears to be a good description for the reactivity pattern of NK cells and related natural effector cells. However, it is noteworthy that, at the time of the Workshop, just five years ago, there was very little direct discussion of natural cell-mediated cytotoxicity.

Concurrent with the initial recognition of cytotoxic reactivity by normal human donors were similar observations in rodent systems. When spleen cells from young, 6-8 week old normal rats were tested as controls for studies of immunity to a Gross virus-induced leukemia, they were found to frequently give substantial levels of lysis above the medium control and often as high as those from tumor-immune rats. After this natural reactivity was found to be a consistent phenomenon (Nunn *et al.*, 1973), similar observations were made with normal mice and initially reported at the Second International Immunology Congress, July 1973 (Herberman *et al.*, 1974). Within the next two years, the initial characterization of mouse and rat NK cells was completed (e.g., Kiessling *et al.*, 1975a,b; Herberman, 1975a,b; Nunn *et al.*, 1976).

As a result of the above reports, another international conference on immunity to human tumors (Stevenson and Laurence, 1975), and two editorials in J. Natl. Cancer Inst. (Baldwin, 1975; Herberman and Oldham, 1975), during 1975

awareness of the limitations in the search for specific disease-related cell-mediated cytotoxicity and of the possible role of natural effector cells became widespread. Thus began a rapid expansion of research activity on natural cell-mediated immunity, with particular emphasis on characterization of the effector cells and on determination of the possible *in vivo* role of these cells (reviewed by Baldwin, 1977; Herberman and Holden, 1978; Kiessling and Haller, 1978; Möller, 1979).

The field has moved more rapidly with mice and rats than with humans, probably because of the availability of many markers for characterization of lymphocyte subpopulations and of inbred strains for analysis of genetic factors and because of the ability to readily perform in vivo manipulations. A major advance in our understanding of the regulation of NK activity began with the observations that inoculation with a variety of stimuli (Herberman *et al.*, 1976, 1977; Wolfe 1976) led to a rapid augmentation of NK activity. When poly I:C, a well-known inducer of interferon was found to have this activity in rats (Oehler *et al.*, 1978), the possible mediation of the augmentation by interferon was suggested. This possibility was soon thereafter confirmed for mouse (Gidlund et al., 1978; Djeu *et al.*, 1979) and human (Trinchieri and Santoli, 1978; Herberman *et al.*, 1979) NK cells.

The progress in this field has been remarkable during the approximately eight years of its existence. Cytotoxicity by cells from normal individuals has evolved from an overlooked or maligned, undesirable effect (thought to be an artifact) to a well-studied area of research. It is rather ironic how great the shift has been, from an almost exclusive emphasis on specific cell mediated immunity against tumor associated antigens to a predominance now, at least for human studies, of reports on NK and other natural effector cells and very few continuing reports of disease-related cytotoxic effector cells.

The current status of the field is well summarized by the contributions in this book. However, it should be clear that there are still more unresolved problems than there are clear answers. Perhaps this book will provide another milestone in this field, and stimulate more extensive efforts to definitively characterize the effector cells and determine their biological roles.

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CHARACTERISTICS OF NK AND RELATED CELLS

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A. Characteristics of NK Cells and Related Cells

1. General

Many reports have eventuated demonstrating disease-related anti-tumor cytotoxic activity of lymphocytes of tumor patients (1-4). Documentation of NK cytotoxicity against a wide variety of cell lines of lymphocytes derived from both normal donors and cancer patients seriously questioned the validity of determination of disease-related cytotoxicity of cancer patients' lymphocytes (5-7).

Although these spontaneous cytotoxic reactivities were initially considered as "undesired background", it was then suggested that this NK cytotoxicity, taking place without deliberate sensitization against notably malignant cells, may serve as an alternative immune surveillance mechanism.

Antibody dependent and/or antibody independent cell-mediated cytotoxicity operate in healthy donors and, for instance, in cancer patients. The former effector cells, which are involved in antibody dependent cell-mediated cytotoxicity (ADCC) against IgG-coated mouse mastocytoma cells (K-cell cytotoxicity), express receptors for the Fc portion of IgG (IgG-FcR). Monocytes have also been show to bear these FcR. A

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number of cell types have been determined to exert the "spontaneous" or NK cytotoxicity in man, such as IgG-FcR bearing cells (8, 9), complement receptor bearing cells (10), carrying FcR as well as complement receptors (11), and activated T-cells (12).

Thus, substantial information is available in the literature to favor the following possibilities:
1. IgG-FcR are present on both NK-cells cytotoxic against monolayer target cells (8, 12) or K-562 cells (13) and K-cells (8, 14-17);
2. soluble factors or antibodies are involved in NK cell killing (18, 19);
3) (a proportion of) NK cells belong to the T-cell lineage of lymphocytes (13, 20, 21). The suggested involvement of antibodies would implicate that the NK cytotoxic cell mechanism is of an ADCC type.

The experiments described here aimed to address the following questions: 1) are the characteristics of the cells exerting NK cytotoxicity against the K-562 erythroleukemic cells growing in suspension (as measured in a short term ^{51}Chromium release assay), the same as those active against human tumor derived cell lines growing in monolayers (as measured in a longterm cytotoxic assay)?; 2) are NK and K reactivities both antibody dependent and possibly displayed by identical cells?

METHODS

1. Purification of mononuclear cells.

Lymphocyte purification: Mononuclear cells were obtained from healthy donors by Ficoll-Isopaque centrifugal sedimentation of heparinized blood. Contaminating phagocytic cells in the isolated lymphoid cells were removed by treating the cells with carbonyl iron plus magnetism. Table I shows the percentage cell yield of the cell separations.

Isolation of sheep red blood cell (SRBC) rosette forming cells: E-RFC enriched and E-RFC depleted populations were obtained. The technique has been described in detail (13, 20, 21).

Separation of cells lacking the IgG-Fc receptor (IgG-FcR) and recovery of cells with IgG-FcR: IgG coated SRBC (EA-RFC) were eliminated by adsorbing these IgG-FcR positive cells to antibody-antigen immune complex monolayers. The technique described by Kedar et al. (22) with some modifications (21,

TABLE I

CELL YIELD EXPRESSED AS PERCENTAGE OF THE INITIAL LYMPHOCYTE NUMBER (% total yield) ± SE AND PERCENTAGE OF THE LYMPHOCYTE NUMBER USED FOR A PARTICULAR SEPARATION STEP (% step-wise yield) ± SE

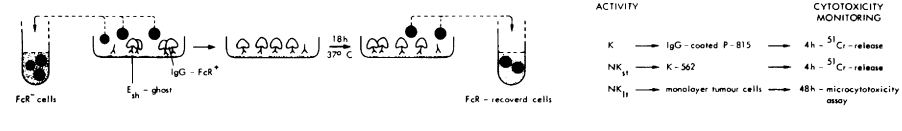
Lymphocyte fractions	% total yield ± SE	% step-wise yield ± SE	no. of experiments
Ficoll-Isopaque purified lymphocytes	100	100	7
Lymphocytes plus iron and magnetism treatment	80 ± 3.1	80 ± 3.1	10
E-RFC enriched fraction (T cell fraction)	55 ± 7.0	68 ± 4.8	6
E-RFC depleted fraction (non-T cell fraction)	7 ± 1.3	8 ± 1.4	6
Direct EA-RFC depleted fraction	63 ± 6.3	64 ± 9.3	12
Direct EA-RFC recovered fraction	21 ± 4.3	23 ± 6.8	1

23) was used. The principal of the technique is depicted in Scheme 1.

Preparation of pure lymphocytes: Ficoll-Isopaque separated lymphocytes were further purified by passing the cells over a nylon column. The procedure used has been described (7). In short, the cells were placed onto a column of unstained spun nylon with a length of 5 cm and a diameter of 15 mm, which had been prewashed with medium supplemented with 5% fetal calf serum. The column was then incubated for 30 min at 37° C. The lymphocytes were eluted with RPMI-1640 plus 5% fetal calf serum, and the eluted cells were washed in medium.

Preparation of monocyte enriched fraction: Monocytes were prepared from Ficoll-Isopaque purified mononuclear cells. One milliliter leukocyte suspension (30 x 10⁶/ml) in RPMI-1640 1640 plus 20% fetal calf serum was added to a Falcon petri dish (60 x 15 mm) and cells were allowed to adhere to the

Scheme 1



surface by incubating the cells for 1 h at 37° C. After washing the plates thoroughly to remove nonadherent cells, the adherent cells were collected by gently scraping the plates with a rubber policeman. The percentage monocytes present in this recovered fraction was 50-60% as judged by electronic sizing (24) and counting of non-specific esterase positive cells (25).

Target cells: P-815-X2 mouse mastocytoma cells and K-562 erythroid leukemic cells were maintained as suspension cultures and used as target cells in a 4 h ^{51}Cr release assay assay (20, 21,26). Colon carcinoma cells and two melanoma cells (Mel-I and NKI-4) were cultured as monolayers and used as target cells in the Takasugi-Klein cytotoxicity assay (26).

It should be noted that the data presented in this section were obtained using cryopreserved lymphoid cells as effector cells. This was due to the fact that for the simultaneous analysis of functional and morphological (i.e. cell surface markers) of unfractionated and fractionated populations, 500 x 10^6 lymphocytes per donor were needed. After the cells were thawed they were incubated overnight before they were used (20, 21).

Determination of T cells by rosette formation with SRBC and anti-T-cell serum, of EA-RFC and of B cells by immunofluorescence after staining the cells with FITC-labelled goat anti-human Fab (Ga/Hu/Fab, Nordic, Tilburg, The Netherlands) has been described in detail (27).

NK_{st} and killer (K) cell antibody dependent cell-mediated cytotoxicity (ADCC): NK_{st} (short-term) and K cytotoxicity were measured in a 4 h ^{51}Cr-release assay using K-562 and P-815 sensitized with anti-P-815-IgG, as target cells (20, 21).

The 48-h microcytotoxicity test has been described by Takasugi and Klein (26). This assay was used to determine the cytotoxic effect of NK cells against monolayer target cells. These effector cells were operationally defined as NK_{lt} (long-term) cells.

RESULTS

1. NK and K (ADCC) activities of unseparated mononuclear lymphoid cells and IgG-FcR negative lymphoid cells:

The results of EA-RFC-separation studies (20,21) are presented in Table II. The aim was to resolve the question

TABLE II

PER CENT CYTOTOXICITY OF VARIOUS LYMPHOCYTE FRACTIONS

	per cent reduction of target cells						immunofluorescence				
Lymphocyte donor	NKI-4	NK_{lt}[1] Mel-1	colon	NK_{st}[2] K-562	K[3] P-815	% E-RFC	% T^+	% Ig^+	% T^+/Ig^+	% T^-/Ig^-	% EA-RFC
Donor A											
1) unfractionated lymphocytes	37*[4]	25*		20*	14*	71[5]	73	8	1	18	12
2) EA-RFC depleted fraction	33*	11		4	0	76	89	1	5	5	1
Donor B											
1) unfractionated lymphocytes	64*		44*	35*	39*	82					22
2) EA-RFC depleted fraction	18		28*	4	5	87	93	1	0	6	0
Donor C											
1) unfractionated lymphocytes		22*	44*	24*		76					20
2) EA-RFC depleted fraction	19*			2	0	93					1
Donor D											
1) unfractionated lymphocytes	35*		31*	47*		74	80	8	2	10	17
2) EA-RFC depleted fraction			35*	0	0	77	98	1	0	1	0
Donor E											
1) unfractionated lymphocytes	15		48*	14*	23*	80	87	6	1	6	20
2) EA-RFC depleted fraction	- 3		4	4	0	96	96	0	0	4	0

1 NK_{lt}: NK cell cytotoxicity against monolayer cultures (48 h MCT)
2 NK_{st}: NK cell cytotoxicity against K-562 (4 h ^{51}Cr-release assay)
3 K_{st}: K cell cytotoxicity against IgG-coated P-815 (4 h ^{51}Cr-release assay)
4 * p value $\leq$ 0.05
5 lymphocytes were not treated. The average yield of 2, 43 % of 1.

whether all NK cells carried FcR on their surface, irrespective of their target cell type. The unfractionated mononuclear cells were cytotoxic against all monolayer tumor target cells and K-562 target cells and displayed K-cell (ADCC) activity. When the EA-RFC were eliminated from the mononuclear cells, no NK_{st} cytotoxicity against K-562 target cells or K (ADCC) cytotoxicity against IgG-P-815 target cells was observed in a 4 h ^{51}Cr-release assay.

The EA-RFC depleted fraction of a number of individuals still displayed cytotoxicity against monolayer target cells in the long-term microcytotoxicity assay (Table II). These results indicate that NK cells in human peropheral blood represent a heterogeneous population of effector cells (20, 21, 28).

The NK_{lt} effector cells are heterogeneous among themselves since elimination of the EA-RFC from the mononuclear cells may a) reduce the level of cytotoxicity against one monolayer target cell but not against the other (Table II, donor A); b) reduce the level of cytotoxicity against both monolayer target cells (Table II, donor B). Apparently, the NK_{lt} consists of both IgG-FcR positive and IgG-FcR negative cells and, moreover, there appears to be selectivity in the recognition and killing of mononuclear cells, also after elimination of IgG-FcR positive effector cells.

The efficacy of EA-RFC depletion is demonstrated by our data showing that no ADCC and no EA-RFC (Table II) are observed after elimination of IgG-FcR positive cells.

One possibility to explain the cytotoxicity of IgG-FcR negative cells against monolayer target cells would be that IgG-FcR are regenerated during the 48 h time period which is needed in the NK_{lt} assays. Regeneration of FcR under certain conditions has been reported (29). This possibility could be excluded since EA-RFC effector cells, being cytotoxic against monolayer target cells in a 48 h microcytotoxicity assay were not able to lyse monolayer target cells or IgG-P-815 target cells in a 4 h ^{51}Cr release assay after prior incubation for 48 h of these effector cells on monolayer target cells (unpublished results).

As for the presence of IgG-FcR on most NK cells, especially those cytotoxic against K-562 this finding is in agree-agreement with data reported by others (8, 9, 11, 30, 31, 32).

2. NK and K (ADCC) activities of E-RFC enriched and E-RFC depleted cell fractions:

The majority of reports indicated that NK cells belong to a so-called non-T "null" cell fraction. This was concluded on

the basis of cell separation studies and subsequent determination of percentages T and non-T cells in the isolated fractions. Table III gives NK_{lt}, NK_{st} and K cytotoxicity data of such cell separation studies and the simultaneous analysis of the cellular composition of these isolated fractions. The isolation of the E-enriched fraction was performed under optimal conditions (13, 20, 21, 33). Both T and non-T cell fractions appeared to be cytotoxic against monolayer cell lines as well as K-562 cells (our data; 13). The per cent lysis of unfractionated cells is given in Table II.

Analysis of the lymphocyte subpopulations by means of E-rosette formation indicated that the T-cell fraction was highly enriched in T-cells and the E-RFC depleted fraction contained virtually no T-cells. When, however, an anti-T cell antiserum (21, 34) was used for the identification of T-cells the following results were obtained:

1) the E-RFC enriched fraction contained virtually pure T-cells (Table III);

2) the E-RFC depleted fraction contained a significant percentage of T-cells as shown by the anti T-cell antiserum (Table III).

That this is not due to non-specific binding of TRITC labelled anti-T-cell serum to the FcR of the cells is illustrated by analysis of individual lymphocytes for the presence of (SIg) and T-cell specific antigen using the two wavelength immunofluorescence method (35). This analysis revealed that virtually no double staining is observed in the unfractionated lymphocytes, containing T-cells and B-cells (SIg bearing cells) and the non-T-cell fraction, containing a high percentage of B-cells as would be expected in case of non-specific staining (Table III; % T^+/Ig^+). Our observation indicates that after rosette formation and separation, T-cells do not form E-rosettes as readily as before the E-RFC depletion. This conclusion is supported by the good correlation between the per cent T-cells as determined by E-rosette formation and by immunofluorescence, when performed before fractionated of these cells on Ficoll (Table III, % E-RFC and % T^+). The implications of this finding are important and may explain a number of the apparent contradicting results in the literature. Kiuchi and Takasugi (9) defined the NK-cell as a null cell, i.e. without T and/or B cell characteristics except the presence of IgG-FcR. These authors used E-rosette formation and separation and subsequently checked the purity of the separated fractions by E-rosette formation. West et al. (13) concluded that the T-cells showing the NK-cell activity belong to a subpopulation of T-cells bearing low affinity receptors of E_{sh}. Apparently, the optimal conditions employed for E-rosette formation and separation do not completely prevent the dissociation of low avidity E-RFC, resulting in the

TABLE III

PER CENT CYTOTOXICITY OF VARIOUS LYMPHOCYTE FRACTIONS

	per cent reduction of target cells						immunofluorescence				
Lymphocyte donor	NKI-4	NK^{lt} Mel-1[1]	colon	NK^{st} K-562[2]	K^{3} P-815	% E-RFC	% T^{+}	% Ig^{+}	% T^{+}/Ig^{+}	% T^{-}/Ig^{-}	% EA-RFC
Donor A											
3) T-cell fraction: E-RFC	15*[4]	- 5		34*		89[5]	93	1	0	6	7
4) E-RFC depleted fraction	43*	38*		32*		5[5]	16	30	1	54	39
Donor B											
3) T-cell fraction: E-RFC	15		9	46*	25*	95	90	1	2	9	14
4) E-RFC depleted fraction	55*		33*	35*	19*	3	6	30	0	62	61
Donor C											
3) T-cell fraction: E-RFC		20*	19*	13*		85	96	0	0	4	3
4) E-RFC depleted fraction		13	40*	24*		3	19	45	1	36	50
Donor D											
3) T-cell fraction: E-RFC	17*		40*	21*		84	97	2	0	1	3
4) E-RFC depleted fraction	- 9		38*	34*		3	17	36	0	47	73

1 NK^{lt}: NK cell cytotoxicity against monolayer cultures (48 h MCT)
2 NK^{st}: NK cell cytotoxicity against K-562 (4 h ^{51}Cr-release assay)
3 K^{st}: K cell cytotoxicity against IgG-coated P-815 (4 h ^{51}Cr-release assay)
4 * p value ≤ 0.05
5 lymphocytes were not treated. The average yield of 3, 55 % of 1; of 4, 7% of 1.

appearance of T-cells with low affinity receptors for E_{sh} after this separation procedure in the interface (21, 36). Since these low-affinity E-RFC have been shown to exhibit the strongest NK-cell activity (13) this could explain the fact that lymphocytes in the non-T-cell fraction, containing only 6-17% T-cells, show a similar level of NK-cell activity as the T-cell fraction (containing virtually pure T-cells, see above) at the same lymphocyte target cell ratio: the latter is relatively depleted and the former relatively enriched for T-cells (NK cells) which bear low-affinity receptors for E_{sh} (our data, 20, 21).

Hersey et al. (12) showed T-cells, bearing FcR, to be cytotoxic. Our data clearly demonstrate that other techniques than the one used for the separation of a cell subpopulation in order to demonstrate the purity of that isolated subpopulation. Thus not all T-cells form E-rosettes after E-RFC separation on Ficoll (36) and a proportion of these T-cells may bear FcR (33, 37). Hence, the cells from the interface, showing NK-cell activity would be characterized as non-T, non-B, IgG-FcR bearing cells on the basis of their E-, EA- and SIg markers. Analysis of cells in this interface fraction with the anti-T cell antiserum, however, proved the presence of T-cells. The simultaneous analysis of cell surface markers and cytotoxicity testing also demonstrated that T-cells (E-RFC, anti-T cell serum positive) can form EA-rosettes and exert K-cell activity (ADCC) confirming data of others (23,37).

The T cell nature of the NK cells was further confirmed by Kaplan and Callewaert (38). These authors succeeded to abrogate NK cell activity by pre-treating the effector cells with anti-T cell antiserum plus complement. Furthermore, significant fraction of NK cells express a receptor for helix pomatia and this receptor is considered to be a T cell marker (39).

The E-RFC depleted fractions are strongly enriched for SIg positive cells (B) (Table III) (7, 21) without a concomittant rise in NK activity, confirming reports by others that human NK cells lack SIg (20, 21, 32). The data presented in Table IV demonstrates that monocytes do not display cytotoxicity themselves i.e. the monocyte enriched fraction exerting the lowest per cent lysis of target cells. Furthermore, human NK cells lack adherent and phagocytic properties (12, 13, 21, 40) and the majority of NK cells do not carry complement receptors (13, 41).

From the data discussed so far it can be concluded that the various NK cell reactivities can be observed and that these NK cells may differ with respect to their characteristics:

1) NK cells displaying cytotoxicity against monolayer target cells and against K-562 suspension target cells as tested in

TABLE IV

PER CENT NK-CELL ACTIVITY OF PURE LYMPHOCYTES WITH AND WITHOUT THE ADDITION OF MONOCYTES, AND/OR A MONOCYTE ENRICHED FRACTION AGAINST NKI-4, Mel-I AND K-562.

Effector cells	Target cells NKI-4[1]	Mel-I[1]	K-562[2]
Nylon purified lymphocytes	39*	83*	47*
Nylon purified lymphocytes + 1% monocytes	49*	78*	50*
Nylon purified lymphocytes + 5% monocytes	58*	83*	48*
Monocytes enriched fraction[3]	27*	69*	19*

1 NK_{lt} : NK cell cytotoxicity against monolayer cultures (48 h MCT)
2 NK_{st} : NK cell cytotoxicity against K-562 (4 h ^{51}Cr-release assay)
3 The monocyte enriched fraction contained 60% monocytes
* p value 0.05

long-term and short-term cytotoxicity assays respectively. These NK cells express IgG-FcR (NK_{lt} and NK_{st}, IgG-FcR positive);
2) NK cells displaying NK cytotoxicity against monolayer cultured target cells exclusively tested in a microcytotoxicity assay, with no demonstrable membrane IgG-FcR: EA-RFC and ADCC negative;
3) NK cells belong, at least in part, to the T cell lineage.

The investigation reported here and by others illustrate that human NK cells are heterogenous and cannot easily be distinguished from K-cells which are present in the same cell fractions as NK_{lt} and NK_{st} cells. One exception was described here, i.e. the IgG-FcR negative cell fraction comprising the IgG-FcR negative NK_{lt}. Thus, NK cell lysis may be of an ADCC type of reaction. Our data concerning the mechanism of NK cytotoxicity in relation to ADCC will be presented in section I.B (this issue).

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ALLOANTISERA SELECTIVELY REACTIVE WITH NK CELLS: CHARACTERIZATION AND USE IN DEFINING NK CELL CLASSES

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I. INTRODUCTION

The phenomenon whereby lymphoid cells derived from the lympho-hemapoietic organs of non-immune laboratory animals and human subjects lyse normal and neoplastic cells *in vitro* has been termed natural killing, and the effector cells natural killer or NK cells (Herberman et al., 1979, Kiessling and Wigzell, 1979). The broad range of susceptible targets and the associated variations in the conditions under which optimal degrees of *in vitro* lysis have been observed have suggested a heterogeneity among the effector cells, and, indeed, recent observations from a number of laboratories have provided evidence for such a heterogeneity. The purpose of this review is to summarize investigations performed in this laboratory on the development of NK specific alloantisera and their use with other antisera which has shown a heterogeneity of NK cells (Burton and Winn, 1980a; 1980b), and to relate these findings to those of others.

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II. METHODOLOGY

The mice used in the studies reported herein were purchased from Jackson Laboratories, Bar Harbor, Maine, and the antisera and methodology used in the experiments have been reported in detail elsewhere (Burton et al., 1978a, Burton and Winn, 1980a; 1980b). The tumor cell lines employed were as follows: YAC, an A/Sn strain Moloney virus induced T lymphoma; EL-4, a C57BL/6 carcinogen induced T lymphoma (T-LYM), P-815, a carcinogen induced mast cell tumor (MST) and WEHI-164, a carcinogen induced BALB/c fibrosarcoma (FSA). Their origins have been dealt with elsewhere (Burton et al., 1977; Burton and Winn, 1980a). Monoclonal anti-Thy 1.2 IgM antibody was purchased from New England Nuclear, Boston, Massachusetts.

III. ALLOANTISERA WITH ANTI-NK ACTIVITY

A. Definition of NK Specificity

In 1977 Glimcher et al. reported that a C3H anti-CE alloantiserum contained specific anti-NK activity in addition to anti-Ly 1.2 activity. This NK specific alloantigen has subsequently been designated NK 1.1 (Cantor et al., 1979). Two further NK specific alloantisera have been defined in this laboratory using the following criteria.

1. Backcross Genetic Analysis. The anti-NK alloantiserum must contain anti-NK activity that segregates separately to any cytotoxicity as detected by the trypan blue test in a backcross analysis of spleen cells from (NK susceptible x NK resistant) F1 x NK resistant mice. Alloantisera made between H-2 compatible mice of different strains potentially contain many different antibodies, and can probably never be regarded as monospecific. NK cells are so small a subpopulation of the spleen (1-2%) that they should not be detected in the trypan blue test (Kiessling and Wigzell, 1979; Dr. N. L. Warner, personal communication). Therefore, the analysis should contain individual mice whose spleen cells are not killed above the complement (C) background, and yet NK activity is removed by treatment with anti-NK serum and C.

2. Functional Tests. In addition to the above, functional tests which indicate that an anti-NK alloantiserum and C does not remove T and B cell activity from a spleen cell suspension for which it does abolish NK activity are also employed.

3. Strain surveys. Strain surveys may provide supplementary evidence to the backcross analysis if strains can be found against whose spleen cells the alloantiserum has anti-NK activity, but for which there is little or no cytotoxic activity in the trypan blue test.

Although all these tests may indicate that an alloantiserum contains only anti-NK activity against a particular strain, it is still possible, and, indeed, even likely, that other antibodies which are non-cytotoxic with rabbit C and/or are directed at other subpopulations, both defined (e.g. macrophages) and as yet undefined, are present in the serum. The usefulness of these alloantisera is not, however, necessarily impaired by the presence of other antibodies, so long as the experimental conditions under which they are used take account of this.

B. Analysis of Three Anti-NK Alloantisera

1. Titrations of Three Anti-NK Alloantisera. Three anti-NK alloantisera produced in this laboratory are shown in Figure 1. Spleen cells of susceptible strains have been treated first with various concentrations of antiserum, and then with pretested rabbit C to produce titration curves of residual NK activity against YAC. As can be seen, the CE anti-CBA serum has by far the highest titer, and, indeed, the anti-NK activity of this pool was 1:1024. The reverse immunization, CBA anti-CE, gave a much weaker anti-NK serum which also contained modest amounts of auto-antibody, and thus required absorption prior to use. This serum is probably an anti-NK 1.1 serum (Table I), and low anti-NK activity plus autoreactivity have been encountered by others using anti-NK 1.1 sera (Dr. G. Koo, personal communication). The C3H anti-ST serum was intermediate in activity against NK cells. The four pools of this serum produced to date have had anti-NK titers in the range 1:8 - 1:32, while the four CE anti-CBA pools have titered in the range 1:32 - 1:1024.

2. Backcross Analysis of Two Anti-NK Alloantisera. All pools of CE anti-CBA and C3H anti-ST alloantisera produced to date have mediated high levels of C dependent lysis of spleen cells of the immunizing strain in the trypan blue test, and

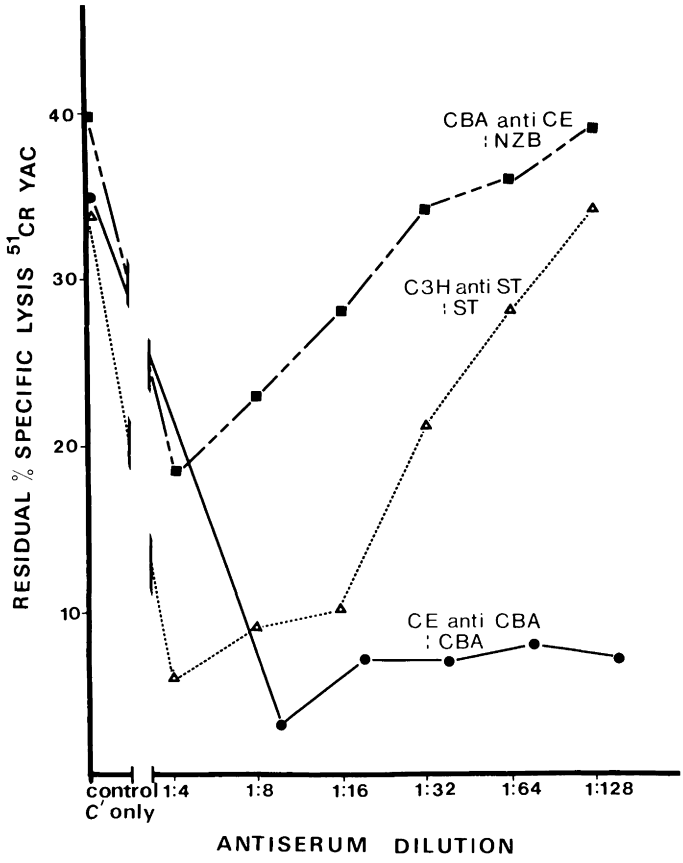

FIGURE 1. Titration of three anti-NK alloantisera.

so clearly contain antibodies in addition to those directed at NK cells. The backcross analyses, however, clearly show that anti-NK activity is separate to the major cytotoxic activity of these antisera (Table II).

a. CE anti-CBA. As can be seen, the cytotoxicity of the CE anti-CBA serum for spleen cells in the trypan blue test segregated quite separately to its anti-NK activity against YAC (Table II - A). These backcross results also indicate that CE anti-CBA serum probably contains two anti-NK antibodies directed against NK specific alloantigens, and that these alloantigens are probably determined by two loosely

TABLE I. Strain Distribution of NK Alloantigens Detected by Specific Alloantisera

	Mouse strains[a]								
Anti-NK alloantisera	CBA	C3H	C57BL	ST	NZB	Ma/My	BALB/c	DBA/2	DBA/1
CE anti-CBA	+	+	+	+	-	-	+	+	-
(NZB x CE)F1 anti-CBA[b]	+	+	+	+	-	-	+	n.d.	-
C3H anti-ST[c]	-	-	+	+	+	+	+	+	+
C3H anti-ST	-	-	+	+	+	+	-	-	n.d.
(C3H x DBA/2)F1 anti-ST	-	-	+	+	+	n.d.	-	-	n.d.
CBA anti-CE	-	n.d.	+	+	+	n.d.	-	-	n.d.
(BALB/c x C3H)F1 anti-CE[d]	-	n.d.	n.d.	+	+	+	n.d.	n.d.	-
Anti-NK 1.1[e]	-	-	+	n.d.	+	n.d.	-	-	n.d.

[a] Murine spleen cells treated with anti-NK alloantiserum and C. Positive if NK activity against YAC was reduced by 50% or more; n.d., not done.

[b] (NZB x CE)F1 anti-CBA antiserum from Dr. N. L. Warner

[c] Some pools of C3H anti-ST serum contain additional anti-NK activity against BALB/c and DBA/2.

[d] (BALB/c x C3H)F1 anti-CE antiserum from Dr. G. Koo.

[e] From Cantor et al. (1979).

linked genes. Functional tests have indicated that at least some of the cytotoxic activity of CE anti-CBA serum in the trypan blue test is probably anti-Ly 1.1.

b. C3H anti-ST. The other backcross analysis (Table II - B) indicated that the C3H anti-ST serum (#36) contained NK specific activity, as defined, because four of the backcross mice were negative in the trypan blue test, yet NK activity was removed from their spleen cells by treatment with C3H anti-ST serum and C. These four mice have subsequently been backcrossed to C3H in order to begin the development of an NK congenic line on the C3H (high NK) background. To date, the fourth backcross mice have been tested with C3H anti-ST serum and C and the NK alloantigen is present, while there is no detectable cytotoxicity in the trypan blue test. The results

TABLE II. Backcross Analysis of Anti-NK Alloantisera

NK alloantigens[a]	*Trypan blue cytotoxicity test*[b]		
	Positive	*Negative*	*Total*
A. CE anti-CBA: (CExCBA)F1 x CE[c]			
Positive	*20*	*15*	*35*
Negative	*15*	*5*	*20*
Total	*35*	*20*	*55*
B. C3H anti-ST: (C3HxST)F1 x C3H[c]			
Positive	*11*	*4*	*15*
Negative	*16*	*2*	*18*
Total	*27*	*6*	*33*

[a] *Spleen cells from individual mice treated with anti-NK + C. Positive if NK activity against YAC was reduced by 50% or more.*

[b] *Spleen cells from individual mice treated with anti-NK + C. Positive if killing of spleen cells above C background.*

[c] *Backcross mice of both sexes individually splenectomized.*

of the backcross analysis also indicate that the NK alloantigen detected by pool #36 C3H anti-ST serum is probably determined by a single segregating locus. Some pools of C3H anti-ST serum, however, contain an additional anti-NK specificity, and there is, as yet, no evidence that this extra specificity is NK specific (Table I).

C. Anti-NK Alloantisera Made in F1 Recipients

On the basis of the findings of Cantor et al. (1979), and of strain surveys performed with the CE anti-CBA and C3H anti-ST defined anti-NK alloantisera, a number of other anti-NK alloantisera have been produced by immunization of appropriate F1 mice. The use of F1 recipients reduces the number of alloantigens by which the strains differ, and thus may reduce the number of antibodies in addition to anti-NK that a serum contains. This is well shown with the (BALB/c x C3H)F1 anti-CE anti-NK 1.1 alloantiserum which, unlike the C3H serum, does not have anti-Ly 1.2 activity (Glimcher et al., 1977). This serum was provided for study here by Dr. G. Koo, and the (CE x NZB)F1 anti-CBA serum was made by Dr. N. L. Warner in a collaborative study. This latter serum appears to sort only NK cells of strain C3H when studied by fluorescence activated cell sorter analysis (Tai et al., 1980), and so may be truly NK cell specific in this strain.

D. Strain Surveys with Anti-NK Alloantisera

The results of strain surveys performed with the NK specific alloantisera in this laboratory have been summarized in Table I. Further genetic analysis of what seems to be a polymorphic system of NK specific alloantigens is currently in progress, and will be the subject of a future review (in preparation). However, it has become apparent that, although NK cells are a very minor subpopulation in lymphoid organs such as spleen, high titer anti-NK alloantisera can be raised when mice are immunized with spleen cells. Thus far, genetic analysis of these sera has indicated that at least two loci which determine NK specific alloantigens are present in NK cells which kill lymphoid tumors such as YAC.

IV. HETEROGENEITY OF NK CELLS

A. *The NK_L Cell and the NK_S Cell*

When fresh murine spleen cell suspensions are treated with either of the two anti-NK specific alloantisera CE anti-CBA or C3H anti-ST and rabbit C, *in vitro* lysis of lymphoma targets is abolished, while lysis of a solid tumor target is not (Table III); thereby defining two different NK cell populations within the one spleen cell suspension: NK_L cells which lyse lymphoid tumor targets and NK_S cells which lyse solid tumor targets (Burton and Winn, 1980b). Based largely on studies of the differential sensitivity to incubation at 37°C of NK cells which lysed solid or lymphoid targets Stutman et al., (1978) and Paige et al., (1978) had suggested that two subpopulations of NK might exist in fresh spleen cell suspensions. The above serological studies now provide definitive evidence for two NK cell types in fresh spleen, NK_L and NK_S cells. Additional experiments with beige mice, which have a near absolute defect in NK activity against YAC (Roder, 1979), have provided further evidence for the two NK cell types (Table III). As can be seen, NK_L activity is virtually absent in beige mice (C57BL/6-bg/bg Jackson Laboratories, Bar Harbor, Maine), while NK_S activity is present.

B. *Do Subclasses of NK_L Cells Exist?*

Although the anti-NK alloantisera have defined NK_L cells as a separate NK cell class, studies on the natural killing of lymphoma targets in other laboratories have suggested that further heterogeneity might exist within NK_L cells.

1. NK_L Cells which Kill EL-4 or YAC. Kumar et al. (1979a; 1979b) have reported that mice treated with the bone seeking radioisotope ^{89}Sr lose NK activity against YAC but retain NK activity against EL-4, and that NK activity against EL-4 appears earlier after adoptive transfer of spleen and bone marrow cells. These studies suggest that two different effector cells are involved at the lytic stage, but do not exclude the possibility that they are both in the same cell lineage. Since both NK cells are sensitive to the CE anti-CBA anti-NK alloantiserum plus C (Table III), it is possible that the EL_4 NK_L cell is a precursor, while the YAC NK_L cell is a more mature cell which is dependent upon an intact bone marrow for its function (Kumar et al., 1979a, 1979b).

TABLE III. Differentiation of NK_L Cells from NK_S Cells

Spleen cell treatment[a]	*Percent specific lysis*[b]		
	EL-4 (T-LYM)	*YAC (T-LYM)*	*WEHI-164 (FSA)*
CBA[c]			
C	*28 ± 1*	*44 ± 1*	*53 ± 2*
CE anti-CBA + C	*0*	*1 ± 0*	*53 ± 2*
NZB			
C		*21 ± 1*	*62 ± 1*
C3H anti-ST + C		*2 ± 1*	*64 ± 2*
BALB/c			
C		*59 ± 1*	*53 ± 1*
CE anti-CBA + C		*4 ± 1*	*49 ± 1*
B6AF1 anti-B10.D2 + C		*6 ± 1*	*44 ± 1*
Rat anti-mouse + C		*4 ± 1*	*29 ± 1*
C57BL/6-bg/bg			
Nil		*1 ± 0*	*46 ± 3*

[a]*Antisera used at 1:4 B6AF1 anti-B10.D2 and rat anti-mouse, 1:10 C3H anti-ST, 1:50 CE anti-CBA; rabbit C at 1:4.*

[b]*Mean ± s.e.m., 1:2 effector dilution after treatment, 16 hour assay, 10^4 target cells.*

[c]*Mouse strain of origin of spleen cells.*

2. Thy-1.2 Negative and Positive NK_L Cells. The question as to whether NK cells which lyse lymphoid targets (NK_L cells) express Thy 1.2 has been a source of controversy for nearly five years, and the advent of monoclonal anti-Thy 1 antibodies has not, as yet, resolved the issue. To date, the controversy has largely revolved around the question of the sensitivity of NK_L cells to anti-Thy 1.2 serum, or monoclonal anti-Thy 1 antibodies, and C, with different laboratories reporting positive and negative results, even when the same monoclonal antibodies were used (Herberman et al., 1979; Kiessling and Wigzell, 1979; Karre and Seeley, 1979; Kumar et al., 1979a; Matthes et al., 1979; Lake et al., 1979). Recent studies using monoclonal anti-Thy 1.2 and the fluorescence activated cell sorter have contributed significantly to this question (Matthes et al., 1979). Their studies indicated that NK_L cells included both Thy 1.2 negative and Thy 1.2 positive subpopulations, and that the relative frequency of each subpopulation was different in nude and normal mice. Thy 1.2

positive NK_L cells predominated in nudes, while the converse was true of normal mice. Since most of the studies referred to above were performed in normal mice, a minor subpopulation of Thy 1.2 positive NK_L cells might not have been detected, especially if C with less than optimal activity was used.

Taken together, these findings suggest that at least three subpopulations of NK cells may exist within the NK_L cell class that lyses lymphoma targets.

C. Other Alloantigens of NK_L Cells

A number of studies have been reported in which alloantisera containing antibodies directed against subpopulations of cells of the lymphohemapoietic system have been used to study NK cells which lyse lymphoma targets *in vitro*. Although the alloantigens which these reagents identify are *not* NK specific these studies have been useful in the investigation of the function and possible cell differentiation pathway of NK_L cells.

1. Ly-5. Ly-5 is an alloantigen which is expressed on most cells of the lymphohemapoietic system (Scheid and Triglia, 1979), including NK_L cells (Cantor et al., 1979; Pollack et al., 1979). In addition, it also appears to be involved in the *in vitro* effector function of NK_L cells, as Cantor et al. (1979) observed that NK activity against a lymphoma target was blocked by an alloantiserum containing anti-Ly 5.1 antibodies. This suggests that antibodies in this serum were binding to structures on the NK_L cell surface very close to or actually involved in target cell recognition and/or lysis. Studies with monospecific anti-Ly 5 reagents might, therefore, provide further insights into the mechanism of NK_L cell recognition of target cell structures.

2. Mph-1. I/st anti-B10.M alloantisera contain antibodies which react with 60% of PEC but not with lymph node cells (Archer and Davies, 1974). This serum, designated anti-Mph 1.2, was used to study promonocyte enriched cultures of mouse bone marrow cells, which are able to lyse YAC *in vitro* (Lohmann-Matthes et al., 1979). It was shown that these cultured cells lost their ability to kill YAC after treatment with anti-Mph 1.2 and C. Although these results suggest that promonocytes might be involved in natural killing, additional studies are clearly necessary. It is possible that anti-Mph 1.2 serum also contains anti-NK_L activity as a separate specificity, and the tissue distribution of Mph 1 itself has not been sufficiently characterized to be certain that it is restricted to cells of the macrophage/monocyte lineage (Archer

and Davies, 1974). The promonocyte enriched effector populations tested also contained about 20% granulocytes (Lohmann-Matthes et al., 1979), and so it is possible that the modest levels of NK activity detected were mediated by granulocytic cells or a minor subpopulation of NK_L cells present in cultured bone marrow.

D. Is NK_S Activity Cell Mediated?

Paige et al., (1978) found that natural cytotoxic activity (NC) against solid tumor targets (NK_S activity) was *not* abolished by treatment of spleen cells with a variety of alloantisera and C. Investigations performed in this laboratory confirm those findings (Burton and Winn, 1980b), but, in addition, show that NK_S activity can be reduced by treatment of spleen cells with high titer rat anti-mouse serum and C (Table III). Of particular interest has been the finding that NK_S activity survives treatment of a spleen cell suspension with anti-H-2 alloantiserum and C, which usually kills all but 1-3% of the cells, including all NK_L cells (Table IV).

In order to be certain that NK_S activity was cell mediated, these 1-3% of live cells were separated from the dead cells by density gradient centrifugation. When tested, the small number of live cells did contain all the NK_S activity. In addition, culture supernatants of fresh spleen cells were tested and, although they had some activity against solid tumor targets, this was very much less than that of cells assayed for the same period of time. Therefore, the NK_S cell seems to be a non-adherent cell, to be deficient in the expression of a number of cell surface antigens and/or resistant to C, to be more resistant to *in vitro* culture than the NK_L cell, and to lyse only solid tumor targets.

E. The NK_C Cell

1. Killer Cells Develop in Unstimulated Spleen Cell Cultures. *In vitro* studies of the weak cytotoxic T cell (Tc) response to syngeneic tumor cells have focused attention on the generation of cytotoxic effector cells (CL) in unstimulated spleen cell cultures (Burton et al., 1978b). It has been observed that, while the effector cells recovered from stimulated cultures are highly sensitive to anti-Thy 1.2 serum and C treatment, those CL recovered from unstimulated cultures of the same spleen cell pool may show only partial sensitivity to the same treatment (Burton et al., 1977). Furthermore, the tumor target used in the ^{51}Cr release assay

TABLE IV. Properties of NK_S Cells[a]

Effector cells	Treatment	% VCR[b]	Percent specific lysis	
			YAC (T-LYM)	WEHI-164 (FSA)
BALB/c	Nil		51 ± 1	54 ± 1
	Adherent cell depletion[c]	50	54 ± 1	50 ± 1
	CE anti-CBA + C	35	6 ± 1	54 ± 2
	B6AF1 anti-B10.D2 + C	2	0	51 ± 2
	B6AF1 anti-B10.D2 + C	3	n.d.	41 ± 0
	B6AF1 anti-B10.D2 + C[d]		n.d.	37 ± 1
BALB/c	5×10^5 spleen cells + 10^4 target cells in 200 ul			41 ± 0
Culture Supernatant	from 2.5×10^6 spleen cells in 1 ml			6 ± 0
	from 5×10^6 spleen cells in 1 ml			10 ± 0
	from 10×10^6 spleen cells in 1 ml			11 ± 0
	from 40×10^6 spleen cells in 1 ml			10 ± 0

[a] Antiserum + C treatment and assay conditions as for Table III.
[b] Percent viable cell recovery after treatment.
[c] Adherent cell depletion by 3 passages of 1 hour at 37°C in plastic petri dishes.
[d] Dead cell removal by density gradient centrifugation.

appears to be an important factor in this phenomenon. For some tumor targets the CL activity is totally abolished by treatment with anti-Thy 1.2 serum and C, while for others it is largely unaffected (Burton et al., 1977, Ching et al., 1978).

2. Studies with Anti-NK and Other Antisera. The serological characteristics of the cultured CL which lyse lymphoid and solid tumor targets are shown in Table V. These CL were totally resistant to treatment with CE anti-CBA serum or anti-Thy 1.2 serum and C under conditions in which all NK_L activity of fresh spleen cells and alloreactive Tc activity induced in culture was abolished. In a previous study these CL were defined as cultured natural killer cells or NK_C (Burton and Winn, 1980b). The additional studies shown here with monoclonal anti-Thy 1.2 antibody, however, indicate that two effector populations can be distinguished. Lysis of the YAC and P-815 tumor cells was mediated largely or totally by CL which were sensitive to anti-Thy 1.2 monoclonal antibody and C, while lysis of the solid tumor target was mediated largely by CL which were resistant to this treatment. The former population are probably Tc, although their resistance to treatment with anti-Thy 1.2 serum and C under conditions which kill all alloreactive Tc induced *in vitro* suggests that their surface density of Thy 1.2 and/or their sensitivity to C mediated lysis must be different.

On the basis of these results, NK_C cells should probably be re-defined as those Thy 1.2 negative CL which mediated most of the lysis of the FSA WEHI-164 and some of the lysis of the T lymphoma YAC.

3. Other Killer Cells which Develop in Culture. Four days of spleen cell culture are required before NK_C activity, as defined above, can be detected, and peak activity is on day 6 (Burton et al., 1977). Under a variety of circumstances, however, cytotoxic effector cells can be detected in 1-3 day cultures. Spleen cells which were cultured with tumor necrosis serum developed two peaks of cytotoxic activity, one at 1-2 days, and a second at 6 days (Chun et al., 1979). The 6 day peak effectors were resistant to monoclonal anti-Qa 5 antibody and C and partially sensitive to monoclonal anti-Thy 1.2 antibody and C, and were probably a mixture of Tc and NK_C as described above. The 1-2 day peak effectors, however, were totally resistant to anti-Thy 1.2 monoclonal antibody and C, and very sensitive to anti-Qa 5 monoclonal antibody and C. Since NK cells which lyse lymphoid targets also express Qa 5 (Dr. G. Koo, personal communication) these effector cells may well have been NK_L cells. Experiments with anti-NK alloantisera could settle this question.

TABLE V. Properties of NK_C Cells[a]

Effector cells	Treatment	Percent specific lysis		
		YAC (T-LYM)	P-815 (MST)	WEHI-164 (FSA)
BALB/c anti-CBA[b]	C	47 ± 2		
6 day culture	Anti-Thy 1.2 + C	6 ± 2		
	Monoclonal anti-Thy 1.2 + C	2 ± 1		
BALB/c[c]	C	51 ± 1		
Fresh spleen	CE anti-CBA + C	6 ± 1		
BALB/c	C	45 ± 1	37 ± 1	54 ± 3
6 day culture	Anti-Thy 1.2 + C	45 ± 2	36 ± 2	42 ± 2
	Monoclonal anti-Thy 1.2 + C	13 ± 1	0	35 ± 2
	CE anti-CBA + C	47 ± 1	31 ± 2	50 ± 2
	B6AF1 anti-B10.D2 + C	n.d.	n.d.	11 ± 1
	Rat anti-mouse + C	n.d.	n.d.	7 ± 1
	Pretreat CE anti-CBA + C[d]	37 ± 3	23 ± 5	41 ± 1
	Pretreat B6AF1 anti-B10.D2 + C[d]	7 ± 3	n.d.	36 ± 1

[a] Antiserum + C treatment and assay conditions as for Table III.

[b] Alloreactive Tc induced *in vitro* as positive control for anti-Thy 1.2 serum.

[c] Fresh spleen (NK_L) cells as positive control for CE anti-CBA anti-NK serum.

[d] Same spleen cell pool treated as shown before culture; controls showed abolition of NK_L activity and preservation of NK_S activity after treatment (day 0).

CL with a spectrum of target activity like that of NK cells in fresh spleen have been detected early in the course (1-3 days) of mixed lymphocyte cultures (Karre and Seeley, 1979). These effector cells, unlike NK_L or NK_S cells, were relatively sensitive to treatment with monoclonal anti-Thy 1.2 antibodies and C, suggesting that they were Tc rather than NK cells.

F. The Relationship Between NK_L, NK_S and NK_C Cells

The NK_C cells are clearly differentiated from NK_L cells by their target preference and resistance to CE anti-CBA allo-antiserum and C; however, their relationship to NK_S cells is less clear. They were highly susceptible to treatment with anti-H-2 or rat anti-mouse serum and C, but this could represent a development of C sensitivity by culture of NK_S cells. However, the NK_C cells, in contrast to the NK_S cells, also killed the lymphoma target. When spleen cells were cultured following treatment with CE anti-CBA serum and C, normal levels of cultured CL activity against both lymphoma and solid tumor targets were observed (Table V), indicating that Tc and NK_C do not arise directly from NK_L. When the 1-3% of spleen cells which survived treatment with anti-H-2 serum and C were cultured, it appeared that only NK_C developed (Table V). The results suggest, but do not prove, that NK_C develop from NK_S.

G. Comments and Conclusions

On the basis of the studies reviewed herein it seems certain that the phenomenon of natural killing is mediated by more than one NK cell type. A minimum of three NK cells (NK_L, NK_S and NK_C) can be differentiated from each other on the basis of target preference, the expression of cell surface antigens and the effects of culture. The effect of culturing spleen cells seems quite variable. Short term culture (24 hours) destroys NK_L cells but not NK_S cells, and long term culture (6 days) generates at least two effector populations, NK_C cells and Tc. Culture in the presence of tumor necrosis serum generates a new NK cell type in 1-3 days, which can be clearly differentiated from the NK_C cell on the basis of the expression of Qa 5. Its relationship to the NK_L cell or NK_S cell is not yet known.

Further studies of the cell lineage(s) to which NK cell(s) belong, other possible *in vitro* functions such as ADCC against lymphoid targets, and their possible *in vivo* relevance should take account of this heterogeneity of the effectors of natural

killing. For example, studies in this laboratory have shown that the effectors of ADCC against chicken red blood cells do not express NK specific alloantigens, while those that mediate ADCC against lymphoid targets do, suggesting that they are NK_L cells (Hamilton, et al., manuscript in preparation). The discovery of specific alloantigens of NK cells of a particular class should enable further dissection of the phenomenon of natural killing to proceed both rapidly and with precision.

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ASIALO GM1 AND THY 1 AS CELL SURFACE MARKERS OF MURINE NK CELLS

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Natural killer (NK) cells were originally defined as a distinctive cell type because they lacked those surface markers characteristic of mature T cells, B cells and macrophages (1). In the six years since this definition, there have been intensive efforts to define the cell surface characteristics of NK cells. These efforts have had two principal goals: (i) documentation of the lymphoid lineage of NK cells and (ii) characterization of a membrane marker confined to NK cells. The latter would have obvious usefulness in the isolation of homogeneous NK cell populations and in determining the identity and contribution of NK cells to the activity of heterogeneous cytotoxic cell populations.

The question of the lineage of NK cells has stimulated considerable interest, and there have been several suggestions that NK cells represent a pre-T cell population. Such proposals have been based principally on the ability of some anti-Thy 1 sera to reduce NK reactivity (2, 3). The validity of this argument has been undermined by the failure of several investigators to affect NK cell activity with anti Thy 1 sera (4). Whether such discrepancies reflect differences in the specificity of the antisera employed or differences in the NK cell populations used has not been satisfactorily resolved. We have addressed this issue by testing the sensitivity of BCG-induced NK cell populations of C57BL/6 mice to treatment with a number of anti Thy 1 reagents. The antibody preparations included conventional AKR anti C3H thymocyte serum and the products of two hybridomas with anti-Thy 1 reactivity.

In search of a membrane marker whose display was confined to NK cells, we have focused on a characterization of the neutral glycolipids and gangliosides of murine NK cells. We have done so because different lymphoid cell subpopulations appear to display characteristic glycolipid profiles (5, 6). We thus asked whether NK cells could be differentiated from other cytotoxic cells on the basis of membrane glycolipid display.

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I. EFFECT OF ANTI THY 1 ANTIBODIES ON NK CELL ACTIVITY

Three antibody preparations with anti Thy 1.2 specificity were used in these experiments to explore effects on BCG induced C57BL/6 NK cells (7). Each antibody was tested, in the presence of either guinea pig or rabbit complement, for its ability to reduce, in parallel, the lytic activities of NK cells and of cytotoxic T cells. In each case, reduction of BCG induced NK activity was observed (Table I). This reduction was dependent upon the presence of complement, since no change in lytic activity was seen in the presence of antibody alone. It was clear however that the reduction in NK activity was much less than that seen when cytotoxic T cells were treated with the same reagents. Comparison of the titration data (Table I) clearly revealed that the cytotoxic T cells were more sensitive to the two mouse antibody preparations. Interestingly, studies with the rat monoclonal antibody showed less distinction between T and NK effector cell populations. In all cases, at the highest antibody concentrations tested, NK cell activity was reduced, (82, 45, and 59% respectively) but, at the same concentrations, inhibition of T cell activity was considerably greater (100, 84 and 88% (Table I)).

Interpretation of these results requires analysis of the specificity of the reagents employed. Employing a 51-Cr release assay with rabbit serum as a complement source, the conventional serum, raised in AKR mice by injection with C3H thymocytes by the method of Reif and Allen (8), showed no reactivity with AKR or C3H bone marrow or with AKR thymus and spleen, when used at a 1:10 dilution. At a 1:50 dilution, the serum lysed greater than 90% of C3H thymocytes and 35-45% of C3H and C57BL/6 spleen cells.

A lyophilized preparation of a mouse monoclonal IgM antibody (F7D5) was the gift of Dr. E. A. Clark (Regional Primate Center at the University of Washington). The antibody was the product of a cell hybrid derived from the fusion of NS-1/1-Ag4-1 cells of the BALB/c P3 (MOPC 21) plasmacytoma and spleen cells of AKR mice responding to immunization with CBA thymus cells. The anti Thy 1.2 specificity of the antibody produced by this hybridoma was demonstrated using Thy 1 congenic mice, in tests of function and tissue distribution (9). Thus, the antibody abolished T helper cell activity for IgM and IgG responses in vivo and in vitro, ablated suppressor T cell activity, and suppressed proliferative responses to the T cell mitogens PHA and Con A. The antibody had no effect on spleen cell responsiveness to LPS. When its cytotoxic activity towards spleen cells was assessed, fifty per cent of the maximal cytotoxicity was observed at a dilution of 1:1000 in the presence of a guinea pig complement source.

The third reagent used in these studies was a concentrated supernatant from an in vitro culture of a rat-mouse hybridoma (Jlj, a gift of Dr. F. Symington). The hybridoma was produced by the fusion of rat spleen cells (immune to C3H thymus) with cells of the mouse myeloma SP2/0. The resulting hybrids were cloned and the Jlj

TABLE I. Effect of anti-Thy 1 antibodies on NK cell activity

Source of anti-Thy 1 antibody	Source of effector cells	% specific cytolysis of L5178Y target cells by cytotoxic cells after treatment with complement and anti-Thy 1 antibody at the following dilutions 1:20	1:40	1:80	1:160	1:320	C alone
AKR anti C_3H	alloimmune T[a]	0	0	0	7	15	45[e]
	NK[b]	5	5	15	18	25	28
Mouse monoclonal anti-Thy 1.2	alloimmune T[c]	7	4	5	7		44[f]
	NK[b]	40	47	54	55		71
Rat monoclonal anti-Thy 1.2	alloimmune T[d]	6	30		50		50[g]
	NK[d]	15	25		33		37

Effector cells (2-3 X 10^7/ml) were pretreated with the indicated dilutions of anti Thy 1 antibody at 4°C for 30 minutes followed by the addition of C for 45 minutes at 37°C. After washing, these cells were tested for residual cytotoxic activity against L5178Y cl 27v. This target cell is Thy 1.2 negative and was selected for its NK sensitivity. NK cell populations were BCG induced pec from C57BL/6 mice given 10^8 viable BCG organisms 4-5 days earlier. Alloimmune T cells were generated either in vivo (expt. I) or in vitro (expts. II & III) against 10^7 L5178Y cl 27 ascites cells (NK resistant). E:T ratios: [a]100:1, [b]50:1, [c]25:1, [d]30:1. C: [e]normal rabbit serum 1:16 final dilution, [f]1:12 guinea pig serum, [g]1:24 guinea pig serum.

hybridoma was selected for its high toxicity towards (C57BL/6 x C3H)F1 thymocytes at dilutions where no toxicity towards AKR thymocytes occurred.

On the basis of the strain distribution of lymphocyte alloantigens, the possible additional specificities which could be present in AKR anti C3H anti Thy 1.2 serum include Ly 1.1, Ly 3.2, Ly 6.1, Ly 7.1, Ly 8.1 and Ala 1.1. Other investigators have identified antibodies to the predicted Ly 1, 3, and 8 alloantigens in conventional sera raised in an identical manner to that employed here (10). With the exception of Ly 3.2, C57BL/6 mice do not carry the appropriate alleles for these loci. NK cell populations have been tested with anti Ly 3.2 serum plus C and found to be negative for this marker (11, and unpublished results). It is therefore unlikely that the reactivity of the AKR anti C3H thymocyte serum towards C57BL/6 NK cells is due to any known contaminant in this serum.

The studies with monoclonal antibodies with documented specificity for Thy 1.2 further substantiate that BCG-induced NK cells bear an antigenic structure resembling Thy 1.2. The reduction of BCG induced NK activity with the mouse anti Thy 1.2 monoclonal antibody, F7D5, is of interest, as this reagent has been previously employed by Clark et al. (12) and by Mattes et al. (3) with conflicting results. In their studies of NK activity present in nude and normal mice, Clark et al. concluded that neither of these sources had NK cells which bore Thy 1. In contrast, Mattes et al. concluded that Thy 1 was present on the major portion of NK cells present in unstimulated BALB/c nude spleen and upon a more limited fraction of cells from CBA spleen. One hypothesis, which would reconcile these conflicting results and would be consistent with our observations, would be that expression of Thy 1 may be correlated with the "activated" state of NK cells. The nude mice which Clark et al. employed demonstrated a much lower activity than those employed by Mattes et al., which suggests the mice differed in their levels of endogenous stimulation. Our own findings with BCG induced NK cells taken together with the findings of Mattes, imply that "activated" NK cells display a higher density of Thy 1 on their surface than do unstimulated NK cells.

The sensitivity of C57BL/6 BCG-induced NK cells to treatment with anti Thy 1.2 antibodies demonstrates either that these cells express a low density of Thy 1.2 (compared to cytotoxic T cells), or that they express a structure antigenically cross-reactive with Thy 1.2. If the macromolecule on the surface of NK cells is not indeed Thy 1, then it must be closely related to it both antigenically and in its expression, for BCG induced NK cell populations from AKR mice (Thy 1.1) were not susceptible to the anti Thy 1.2 reagents used (data not shown). If this structure is not identical with Thy 1.2, eventually a discordance should be observed when comparing T cell and NK cell sensititivity to several monoclonal anti Thy 1.2 antibodies. With the two anti Thy 1.2 monoclonal antibodies we have used, such differences

were not apparent. The presence of Thy 1 on NK cells is compatible with earlier suggestions that these cells are of the T cell lineage.

II. GLYCOLIPID MARKERS OF NK CELLS

Experiments by Marcus and his colleagues (5, 6) have indicated that lymphocyte subpopulations have distinctive glycolipid profiles. By immunofluorescence, they noted that rabbit antisera raised against ganglioside GM1 reacted with peripheral T cells and thymocytes. On the other hand, antiserum against the unsialated derivative, asialo GM1, reacted with only 30% of peripheral T cells and not with thymocytes. The reactivities of both antisera were independent of the Thy 1 phenotype of the cells.

These observations encouraged us to examine whether NK cells, both endogenous and BCG-induced, might express a particular glycolipid, which would then be useful as an identifying marker. We have assessed the sensitivity of NK cell mediated lytic activity to treatment with a series of rabbit anti glycolipid antisera (13). These experiments were carried out in collaboration with Drs. W. Young, L. Patt and S-I. Hakomori, who kindly provided us with the antisera which they had raised (14).

Pretreatment of peritoneal exudate cells from BCG stimulated mice with anti asialo GM2 or with anti globoside, with or without C, did not alter NK activity (Table II). In striking contrast, however, pretreatment of the effector population with anti asialo GM1 (or with affinity-purified anti asialo GM1), in the presence of C completely eliminated NK activity. This elimination of cytotoxic activity was accompanied by lysis of approximately 15% of the cells in the effector population. Anti ganglioside GM1 reduced NK activity by approximately 25%. Analysis of the specificity of this reagent suggested that this could be explained entirely by cross reactivity with anti asialo GM1 (as previously noted, 6).

Comparative studies were undertaken to define the difference, if any, in membrane antigen display between the uninduced NK cell and the BCG induced NK cell. Figure 1 illustrates that similar reduction patterns are seen in both activities after treatment with anti asialo GM1 and complement. Kasai et al. (15) have also identified asialo GM1 as a marker for uninduced NK cells.

It is important to note that although asialo GM1 was described initially as a marker for cells lacking surface immunoglobulin, and thus presumably for T cells, we and others have found that alloimmune T killer cells were unaffected by treatment with anti-asialo GM1 and C (Figure 1; see also (15)). Thus, anti-asialo GM1 serum appears to be a useful tool in defining a cytotoxic cell as an NK cell. Additionally, it can be used for both positively and negatively selecting for NK cells, since unlike Ly 5 serum (11, 16), anti asialo GM1 serum does not block NK activity in the absence of C (data not shown).

TABLE II. Effect of Anti-Glycolipid Antibodies on NK Activity

Effector cell pre-treatment		NK activity % specific cytolysis at effector to target ratios of:			% cells killed by antiserum
Antiserum directed to:	Complement	45:1	15:1	5:1	
-	-	37.0	20.4	12.0	-
-	+	40.2	22.6	12.4	0
asialo GM_1	-	27.8	14.3	4.0	0
	+	1.0	-1.4	-2.4	18
asialo GM_1 (affinity purified)	-	30.7	16.2	-	5
	+	1.0	-2.7	-3.0	13
GM_1	-	38.6	15.5	9.5	5
	+	28.2	15.1	7.2	1
globoside	-	37.4	20.2	9.3	-2
	+	30.5	19.7	9.6	4
asialo GM_2	-	42.8	17.9	7.0	0
	+	35.6	18.6	8.6	0

BCG induced peritonal exudate cells (4×10^6/ml) were incubated for 30 min. at 4^o with the indicated antisera (1:10 final dilution) followed by addition of native guinea pig serum as complement source (final 1:24 dilution). Incubation was continued for 45 min. at 37^o, after which the cells were washed three times and brought to a constant volume (0.4 ml). The microcytotoxicity assay was performed with 10^4 ^{51}Cr-labeled target cells at the indicated effector:target cell ratio in a final volume of 0.2 ml. After 4 hours incubation at 37^o, 0.1 ml of the cell-free supernatant was assayed for ^{51}Cr content. The percentage of the effector cell population killed by antiserum treatment was determined by vital dye exclusion.

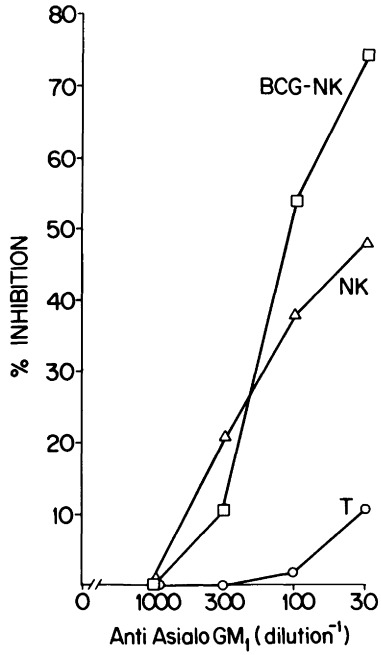

FIGURE 1. Effect of anti asialo GM1 serum on NK cell, BCG induced NK cell and cytotoxic T cell activity. Various effectors were pretreated with anti asialo GM1 at 4°C for 30 minutes followed by incubation at 37°C for 45 minutes with or without the addition of C (guinea pig serum final dilution 1/24). The values shown are relative to lysis after C treatment alone at an effector:target ratio of 25:1. The target is L5178Y clone 27v. The values for specific lysis for untreated, C treatment alone, and anti asialo GM1 (1/30) treatment alone are: 35.8, 33.9, and 35.3 for BCG induced pec of C57BL/6 origin ( ), 14.6, 12.6, and 13.2 for spleen cells from normal CBA mice ( ), and 37.9, 36.6, and 39.3 for alloimmune T cells ( ). BCG pec were harvested 4 days after inoculation i.p. with 10^8 BCG. Alloimmune T cells were generated in 5 day cultures utilizing the NK resistant ascites line as antigen (L5178Y clone 27a).

In our studies comparing the cell surface display of asialo GM1 on endogenous and BCG-induced NK cell populations, we became interested in examining the status of induced NK cell populations from C57BL/6 bgJ/bgJ (beige) mice. Homozygous beige mice are reported to have a lysosomal defect resembling Chediak-Higashi syndrome in man (17) and recently have been reported to lack NK activity (18). Attempts to induce activity with interferon, or the interferon inducers tilorone and poly I:C, were reportedly unsuccessful (18). However, we have found that peritoneal exudate cells harvested from beige mice given BCG i.p. have significant lytic activity (Table III), although no activity was demonstrable in normal peritoneal exudate cells or in normal spleen cell populations from beige mice. This BCG induced activity has been found to reside in the nylon wool non-adherent population and to be resistant to concentrations of anti Thy 1.2 antiserum which eliminated cytotoxic T cell activity but not NK activity (data not shown). In keeping with the data presented in Table I, when using higher concentrations of anti Thy 1.2 serum we ablated the NK activity of beige mice. Additionally, we demonstrated that the cytotoxic activity of beige mice was sensitive to treatment with anti asialo GM1 serum plus complement (Table IV). Thus, although no (or very little) endogenous NK activity could be measured in beige mutant mice, it appeared that the activity which arose upon stimulation with BCG was identical with that in normal mice.

In sum, BCG induced NK cell populations from C57BL/6 mice bear either Thy 1.2 alloantigen, or a macromolecule which antigenically cross-reacts with it to a considerable degree. The density of Thy 1 display on NK cells appeared to be considerably less than on cytotoxic T cells, since all of the antibodies used ablated cytotoxic T cell activity at greater dilutions than those at which they inhibited NK cell function.

The neutral glycolipid asialo GM1 was readily demonstrable on murine NK cells. This was true both for NK cell populations from normal CBA spleen and for BCG-induced NK cell populations from CBA, C57BL/6 and beige mutant mice. In contrast, although 30% of peripheral murine T cells are reported to display asialo GM1, this glycolipid could not be demonstrated on cytotoxic T cells. These findings suggest that asialo GM1 may prove to be a useful marker for the identification and isolation of NK cell populations. Furthermore, investigation of the display of this marker during NK cell ontogeny may prove to be useful in delineating the cellular origin of these cells.

TABLE III. NK activity in beige mutant mice and normal littermates.

PEC Effector cell Source	% specific lysis of YAC-1 cells at E:T 40:1	20:1	10:1
Homozygous beige BCG-induced	28.0 ± 0.4	18.1 ± 1.2	11.6 ± 0.2
Uninduced	--	0.7 ± 0.5	--
Littermates BCG-induced	77.9 ± 1.3	69.4 ± 0.5	58.5 ± 1.4
Uninduced	--	4.0 ± 0.2	--

Specific lysis was measured in a 4 hr 51-Cr-release assay with BCG-induced and normal PEC as effector cells.

TABLE IV. Effect of anti-asialo GM_1 plus complement on beige NK cell activity.

Treatment	% specific lysis of YAC-1 cells by BCG induced PEC from: Homozygous beige	Littermates
Guinea pig serum (C)	13.0 ± 1.1	70.1 ± 0.5
anti-asialo GM_1	14.1 ± 0.2	67.7 ± 1.8
anti-asialo GM_1 + C	-3.0 ± 0.2	20.0 ± 2.0

Specific lysis was measured in a 7 hour 51-Cr-release assay at an effector:target cell ratio of 20:1. The effector cells were pretreated with the antiserum (1:10) and/or C (1:12) and washed before readjustment to equal cell numbers and use in the assay.