CELL-MEDIATED IMMUNITY TO CARCINOMA OF THE URINARY BLADDER

Specificity of the reaction and the nature of the effector cells

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1977
PUBLICATION OF THE RADIOBIOLOGICAL INSTITUTE
OF THE ORGANIZATION FOR HEALTH RESEARCH TNO,
RIJSWIJK, THE NETHERLANDS

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CHAPTER I

INTRODUCTION

A. Immunosurveillance as the rationale of tumour immunological research

The hypothesis that immune mechanisms are a defense system not merely for the elimination of microbes, parasites and other foreign material from the body but also for the destruction of aberrant cells of the organism itself was already clearly formulated in 1909 by Paul Ehrlich: 'were it not for the defense mechanism of the organism tumours would appear "in einer geradezu ungeheuerlicher Frekwenz"'. The theory of immunosurveillance, first mooted by Thomas (1959) was refined and presented in more detail by Burnet (1970; 1971). He postulated that elimination of malignant cells is one of the major functions of the immune system and thought that it may be a very ancient if not the most ancient mechanism. It would have evolved at the time when multicellular organisms first appeared and the need to deal with insubordinate autonomous cell variants may have been at least as important as a protection against foreign substances.

Immunological responses to tumour associated antigens (TAA) are now thought to play an important role in modifying or even controlling human malignant disease. The rationale for this concept is based on the experience obtained with experimental animal tumours induced by oncogenic viruses or by chemical carcinogens. These tumours showed tumour associated transplantation antigens (TATA) which under certain conditions can provoke a tumour rejection response (Bauer, 1974;

Baldwin, 1973). These experiments were carried out in highly inbred strains which ensured that the immune reactions observed were directed against tumour associated antigens rather than histocompatibility antigens. Furthermore, the phenomenon of concomitant immunity could be demonstrated in animals bearing a progressively growing tumour since these animals were capable of rejecting a "small" subsequent graft with limited numbers of cells of the same tumour at a site distant from the progressively growing tumour (Vaage, 1971; 1973).

These findings strengthened the belief that the immune response of the host played a significant role in the tumour-host relationship effecting the tumour growth. This concept of immunosurveillance against neoplastic cells has, however, also seriously been criticised by several investigators (Nehlson, 1971; Prehn, 1971).

The literature on tumour immunology has become vast and this introduction is not an attempt to review the whole field since many excellent reviews on various aspects of tumour immunology have been written (Hellström and Hellström, 1969; 1974a; Klein, 1973; Baldwin, 1973; 1977; Herberman, 1974; Prehn, 1976; Bonnard and West, 1977).

This chapter will discuss the role of the immune system in modifying tumour growth and present literature data discussing the pro's and con's regarding the validity of the concept of immunosurveillance and the types, specificity and immunogenicity of antigens which are associated with tumours of different etiology. The evidence for cell-mediated mechanisms against tumour associated antigens, the specificity of these reactions and the characteristics of the effector cells will be dealt with in the following chapters.

B. Virally induced tumours and immunosurveillance.

1. Virally induced tumour associated antigens and embryonic antigens

Tumours induced by the same oncogenic virus have a com-

mon antigenicity irrespective of their tissue or species of origin, and within the same host strain different oncogenic viruses elicit different tumour associated antigens (Habel, 1961; Sjögren, 1965).

Viral antigens that result from lytic infection of cells with RNA tumour viruses are found on the surfaces of tumour cells and normal cells (Old et al., 1965) and immunity to these antigens can occur dependently (Sjögren et al., 1961) and independently from immunity to the virus particles (Khera 1963), indicating that at least a proportion of these antigens are distinct and different. Furthermore, tumour associated antigens may be found on the surface of virus induced tumour cells which are not tumour-specific and could be present on normal cells during certain embryonic stages. These foetal antigens are found on many tumour cells and will be briefly discussed later. They may appear as a consequence of transformation. It should be noted that these foetal antigens can be detected in small quantities in normal adult tissues (Coggin and Anderson, 1974), and blood levels of these antigens also rise during some non-neoplastic diseases.

2. Experimental evidence for surveillance against tumours induced by oncogenic viruses.

The observations of many workers that treatment with antilymphocyte serum (ALS) or neonatal thymectomy increased the frequency of experimental tumours induced by RNA or DNA viruses in rodents supports the theory of immunosurveillance (Law, 1966a; b).

The restoration of the crippled immune system of neonatally thymectomized or ALS treated animals by thymus grafting or inoculation of syngeneic mature lymphocytes prevented the majority of tumours induced by viruses such as polyoma and SV-40, demonstrating that the increased tumour incidence was specifically due to a T-cell deficiency (Allison and Taylor,

1967).

This experiment does not allow the conclusion that the immune system acted exclusively through killer T-cells but shows that the immune response is in its essence thymus-dependent. One could envisage T-cell dependent antibodies as being responsible for the prevention of tumour growth and this in turn could be either a complement dependent cytotoxic mechanism or a lymphocyte dependent antibody cytolysis.

Polyoma virus is a widespread natural contaminant and yet wild mice do not develop polyoma tumours (Huebner et al., 1962; Huebner, 1963).

Neither did captured animals, when kept in the laboratory for prolonged periods of time, develop tumours. Antibodies with specificity for the virus could be detected in the serum of most of the animals and virus could be isolated from their tissues.

The lack of oncogenicity of polyoma virus under natural conditions and the fact that tumours could only be induced when the virus was inoculated in newborn mice during their first 24 - 48 hours of life favoured the view that the oncogenic properties of the virus were no more than a laboratory artefact. In fact, the contrary is true. Newborn animals are protected from the virus during neonatal life by the passive transmission of antibodies from their infected mothers and are therefore as resistant to the oncogenic effect of the virus as laboratory mice. Indeed the naturally virus-infected newborn mice will only develop tumours if the passive transfer of maternal antibodies is prevented (Sjögren and Ringertz, 1962). The resistance later in life is due to an immediate recognition of the virus induced tumour specific transplantation antigens on the surface of the tumour cells. Furthermore, congenitally athymic nude mice show an increased susceptibility to tumour development after infection with polyoma virus as well (Stutman, 1975a). The fact that immunosuppressed adult mice develop polyoma tumours after exposure to room infection (Law and Dawe, 1960) shows the potential oncogeni-

city of the virus and favours the immunosurveillance to be a real operating mechanism under natural conditions. The polyoma induced tumour specific transplantation antigen was the first virally induced tumour antigen discovered (Sjögren et al., 1961).

Another example sustaining the concept of immunosurveillance is that treatment of AKR mice with anti-lymphocyte serum potentiated murine leukaemia virus induced tumours and shortened the latent period of spontaneous leukaemia (Allison and Law, 1968). Vandeputte et al. (1963) demonstrated that neonatal thymectomy of rats, infected at birth with polyoma virus, increased the incidence of tumours and prolonged the period of postnatal susceptibility to oncogenesis. Similar results were obtained by Allison and Taylor (1967) with SV-40 infected rats. Moreover, polyoma virus infected adult mice showed an increase in tumour incidence from 0 to virtually 100 per cent after anti-lymphocyte serum treatment (Allison and Taylor, 1967; Allison and Law, 1968).

In general, it can be concluded that immunosuppression leads to an increased susceptibility for tumour induction by viruses. It may be assumed that the frequent natural exposure of wild mice to polyoma virus has selected them for a high immune responsiveness both against the virus that lives in symbiosis with them as well as against potentially neoplastic cells carrying the tumour specific transplantation antigen induced by the virus, thereby alerting the appropriate immune rejection system in time to prevent the malignant outgrowth of these cells.

Immune response genes are known to influence virtually every type of immune response (Munro and Bright, 1976) that exists on several levels ranging from the antigen recognition by T-cells (Shevach and Rosenthal, 1975; Rosenthal and Shevach, 1976; Erb and Feldmann, 1975) through cooperation with B-cell antibody formation at the B-cell level (Kindred and Shreffler, 1972; Katz et al., 1973; Katz et al., 1975) to the action of helper and suppressor T-cells (Rosenthal and She-

vach, 1976; Pierce et al., 1976). The differences in immune resistance of different mouse strains against the oncogenic effect of viruses may be explained by the differences in the genetic make-up of the immune response genes. Furthermore, it is known that the cytolysis of virus infected cells by immune T-cells requires at least partial identity of the major histocompatibility complex between the effector T-cell and the target cell (Zinkernagel and Oldstone, 1976; Schröder and Edelmann, 1976), again emphasizing the importance of genetics for anti-tumour immune responses. Genetic make-up influences the level of immunoresponsiveness to a given antigen, the class or type of antibody produced and the timing of the response. The latter is likely to be of extreme importance for the immune response against tumour associated antigens.

C. Chemically induced tumours

1. Chemically induced tumour antigens specific for the individual tumour and embryonic antigens

In contrast to the common antigenicity of virus induced tumours, tumours induced by the same carcinogen and of the same histology, organ, or animal of origin, often possess individual distinctive neo-antigens at their cell surface which are immunogenic in the tumour-bearing host (Prehn and Main, 1957; Klein et al., 1960; Baldwin and Embleton, 1971). The immunogenicity as measured by the degree of resistance to the growth of a challenge inoculum varies from tumour to tumour and ranges from non-immunogenic to highly immunogenic (Main and Prehn, 1957; Prehn, 1960; Old et al., 1962; Bartlett, 1972).

Prehn (1975) demonstrated in the case of 3-methylcholanthrene (MCA) that there is a direct relation between the average immunogenicity of the induced tumour and the concen-

tration of the oncogen i.e., a decrease in the concentration of chemical carcinogen resulted in a lower tumour incidence, a lengthened latency and a decreased immunogenicity. Similar types of new antigens may also be demonstrated on the surface of cells transformed *in vitro* by exposure to chemical carcinogens (Mondal et al., 1970; Embleton and Heidelberger, 1972).

Besides the individual distinctive neo-antigens almost all chemically induced tumours of diverse histological types have been found to express and share embryonic antigens as well. These embryonic antigens are widely expressed in tumours and no relationship to etiology has been demonstrated, i.e. they have also shown to be present on tumours induced by oncogenic viruses and on spontaneous tumours (e.g. arising without an artificial stimulus) (Baldwin et al., 1974a; Baldwin et al., 1974b; Coggin and Anderson, 1974).

The individual distinctive antigens are in general the cell surface components of tumour cells that serve as targets in the immune tumour rejection process since immunization with these antigens provides resistance against the immunizing tumour exclusively (Baldwin and Price, 1977).

2. Chemically induced tumours and the role of immunosurveillance

The potency of the immune system to cope with chemically induced tumours is, however, less impressive than its interaction with tumours induced by viruses in their natural hosts. The influence of immunosuppression produced by for instance treatment with ALS or immunosuppressive drugs on chemical carcinogenesis is equivocal (Baldwin, 1973; Schmähl et al., 1974). Several investigators did not find an effect of neonatal thymectomy on oncogenesis by 3-methylcholanthrene (Balner and Dersjant, 1966; Allison and Taylor, 1967).

The data available on the effect of antilymphocyte serum on tumour induction by 3-methylcholanthrene are conflicting, some authors reporting a slight increase in tumour incidence

and shortening of the latency period following ALS treatment (Balner and Dersjant, 1969; Cerilli and Treat, 1969; Rabbat and Jeejeebhoy, 1970), others reporting no effect (Fisher et al., 1970; Haran-Ghera and Lurie, 1971).

Wagner and Haughton (1971) reinvestigated this problem extensively. They induced prolonged immunosuppression with ALS in mice and reported that no effect of ALS treatment on 3-methylcholanthrene induced tumour incidence, latency, incidence of metastases or antigenicity was observed. The immunosuppressieve potency of the ALS was assayed by transplanting H-2 incompatible tumours and subsequent monitoring of the tumour take in treated and untreated mice. These experiments certainly do not support the concept of immunosurveillance. Studies by Stutman (1974; 1975b), showing that tumour incidences in congenitally athymic nude mice, following exposure to either 3-methylcholanthrene or urethan were not significantly different from those in the normal heterozygous littermates led to the same conclusion. As already mentioned, there is an increase in the susceptibility to the development of polyoma virus induced tumours in this strain of mice (Allison et al., 1975; Stutman, 1975a).

It could be argued that since the chemicals themselves are immunosuppressive (Malmgren et al., 1952; Prehn, 1963; Stjernswärd, 1967) thereby exerting a dual effect at both the host and tumour level during oncogenesis it is almost impossible to dissect the different processes going on. The fact, however, that there is a direct relationship between the dose of oncogen and the immunogenicity of the tumour resulting from it, and its inverse relationship with latency can be explained within the framework of the immunosurveillance theory. The severe immunodepression that results from a high dose of oncogen allows the growth of tumours which are highly antigenic. At lower doses of the oncogen, however, the immune system is less affected and can recover more rapidly. Therefore, immunosurveillance would be more effective so that tumours would appear later and be less immunogenic.

Bartlett (1972) and Parmiani et al. (1973) provided an experimental basis for this hypothesis of immunoselection. If mouse cells, placed in diffusion chambers, were exposed to 3-methylcholanthrene and subsequently transplanted subcutaneously so that at least during the early process of oncogenesis immunoselection was excluded, the correlation between oncogen dose and latency and immunogenicity of the tumour was lost. Similar results were obtained with tumours that arose from cells spontaneously transformed in diffusion chambers (Bartlett, 1972; Parmiani et al., 1973). The tumours that arose from cells transformed *in vitro* by 3-methylcholanthrene were on the other hand highly immunogenic *in vivo*. Prehn (1971) tested the immunogenicity of cells spontaneously transformed *in vitro*. The tumours resulting from these cells were never found to be immunogenic *in vivo*. Therefore, these data might favour an alternative explanation of the results obtained by Bartlett (1972) and Parmiani et al. (1973), namely that the immunogenicity of tumours arising was a function of the presence of the oncogen rather than the result of immunoselection. If otherwise, one would expect the cells transformed spontaneously *in vitro* to result in highly immunogenic tumours as well since in this system immunoselection plays no role. The arguments regarding the immunosurveillance become even more controversial by the observations of immunostimulation, *in vitro* as well as *in vivo*, of tumour growth (Prehn, 1971; Prehn and Lappé, 1971; Fidler, 1973; Medina and Heppner, 1973; Shearer et al., 1973; Bray and Keast, 1975; Kall and Hellström, 1975).

If immunostimulation of tumour growth is real, then its role in tumour biology is of an enormous importance. The nude mice do not provide an answer to this question. The incidence of spontaneous tumours is the same as in the heterozygous littermates (Stutman, 1974; Outzen et al., 1975), with the exception of lymphoreticular tumours and they are equally susceptible to the induction of tumours by the use of carcinogens. Therefore, neither immunostimulation nor immunosurveillance seems to play a role in these two systems. One has

to bear in mind, however, that nude mice have a very efficient B-cell system capable of remarkably efficient responses. Moloney sarcoma virus induced tumours, however, do not regress in nudes as they do in normal mice. The human analogue would be the leprosy patient, who has a severely decreased T-cell activity and yet no increased tumour incidence. Moreover, it should be considered that T-cell independent, humoral immunity, rather than cellular immunity may constitute the main defence mechanism against the persistance of small numbers of malignant cells (van Bekkum, 1975) or that small numbers of cells evoke a primary humoral immune response, whereafter T-cells may lyse the malignant cells in an antibody (IgM) dependent way (see Discussion, chapter IV).

The mechanisms of immunostimulation are unknown and may involve a number of effector mechanisms such as antibodies, T-cells or both and also non-immunologic mechanisms, depending on the conditions and systems studied.

D. Escape from immunosurveillance: Possible mechanisms

The host-tumour relationship consists of two dynamic systems, both potentially capable of adapting to selective pressures from the internal and external environment. The neoplastic cell has an unlimited proliferative capacity. When this cell becomes subject to restrictive conditions which at times do not lead to its elimination, it may circumvent the restriction by producing variants resistant to the selective pressure, maintaining its unlimited proliferative capacity. This may lead to a form of coexistence between the host and the tumour or one may become to dominate and ultimately eliminate the other. The immune response of the organism against the tumour and the escape of the tumour from it are part of this multifaceted relationship. There are several mechanisms to be encountered such as:

1. immunodeficiency of the host,
2. "sneaking-through",
3. immunoresistance, lack of recognition and malfunctioning of the effector cells, and
4. shedding of antigen, antigen-antibody complexes and immunostimulation.

1. Immunodeficiency of the host

Numerous experimental and human tumour systems can be found in the literature where the development of a tumour is attributed to a deficiency of the immune mechanism such as the already mentioned ALS treatment and neonatal thymectomy. Irradiation is another well documented immunosuppressant resulting in a temporarily generalised depression of the lymphoid system and marked lymphopenia (Buckton et al., 1967; Thomas et al., 1971). The increased incidence of a number of autoimmune diseases (Good and Yunis, 1974) has been associated with an age-related decline of the immune capacity. The impaired capacity of the ageing immune system may therefore result in the development of tumours in old age. Makinodan has made an extensive study of the reactivity against experimentally given antigens as a function of age (Makinodan and Peterson, 1964; 1966a; 1966b; Albright et al., 1969; Nordin and Makinodan, 1974). These authors observed an age-related decline in the primary as well as the secondary immune responsiveness. Cell transfer experiments demonstrated the decline of the immune capacity to be mainly due to the cells of the immunological system itself rather than to changes in the environment in which these cells had to function. Brennan and Jaroslow (1975) showed an age-related reduction of the number of theta bearing cells as well as the amount of theta antigens per individual T-cell. The response to phytohaemagglutinin stimulation of T-cells in mice (Hori et al., 1973; Gerbase-DeLima et al., 1975), in rats (Kruisbeek, 1976) and in man (Roberts-Thomson et al., 1974; Weksler and Hütteroth, 1974)

has been reported to be impaired. Also the ability of splenocytes to lyse tumour cells and the ability to reject skin allografts is shown to decline with age (Menon et al., 1974).

Prolonged immunosuppression also results in an increased tumour incidence. Kidney graft recipients which are under prolonged immunosuppression show an approximately 80 times higher incidence of tumours (Penn and Starzl, 1972; 1973). Lymphomas have been prominent and therefore one might argue that immunosuppressive agents acted as a carcinogen apart from their surveillance inhibiting action. The incidence of solid tumours, however, is also reported to be increased. These tumours (about 60 % are skin cancers or carcinoma of the cervix or of the lip) are precisely those who are thought to have a viral etiology (Schwartz, 1974; Melief and Schwartz, 1975). These authors, however, suggested that immunity against an infectious oncogenic virus may be a more relevant question than surveillance against a barely immunogenic stimulation by the graft. Immunosuppression, adequate to eliminate a feed-back regulatory mechanism limiting lymphocytic proliferation could then cause the high incidence of malignant lymphomas (Krueger, 1972; Schwartz, 1972). The association of immunocompetence and tumour incidence has been pointed out by Melief and Schwartz (1975). The overall incidence of malignacies in certain immune deficiency diseases in young children is also highly increased. With one exception (Bruton type agammaglobulinaemia, where all malignancies were leukaemias), again epithelial tumours do arise in other immune deficient states. Again, the preponderance of leukaemias and lymphoreticular tumours may be related to the higher susceptibility to virus infection, if one considers the possibility that these malignancies may be induced by oncogenic viruses as known for many animal species. Van Bekkum (1975) provides an alternative explanation. He states that "these form a high proportion of all malignancies in this age group, so that the immune deficient state would merely provide a

more favourable environment for carcinogenic factors to which all children are exposed".

2. "Sneaking through"

In numerous experimental systems it has been observed that transplantable chemically induced sarcomas did or did not progressively grow depending on cell dose: very small doses of cells growing progressively, relative small doses were rejected and larger doses again grew progressively (Old et al., 1962). Apparently the low dose of tumour cells does not provide an effective antigenic stimulus and the tumour reaches an irreversible size before an immune response is mounted. On the other hand there is some evidence that the phenomenon is due to a specific, partial tolerance, produced by a small inoculum (Bonmassar et al., 1974). This observation resembles that of Stillström (1974), who showed that exposure to a small dose of transplantation antigens results in a low response to that antigen after subsequent stimulation by that antigen, regardless of the dose. A very small dose of tumour cells may mimic both, *in situ* tumour formation and the phenomenon described above. The "sneaking through" hypothesis was originally proposed by Old et al. (1962), and elaborated by Humphreys et al. (1962) and Marchant (1969).

3. Antigen modulation: resistance against immune attack

The changes in the cell surface antigens as a result of malignant transformation are complex and it may be that some neo-antigens, although capable of eliciting an immune response that can be measured *in vitro*, do not function as tumour rejection antigens *in vivo*. Besides, the degree of integration of these antigens in the cell surface may be important. Little is known about their molecular organization and their

association with the membrane lipid bi-layer remains largely to be investigated (Singer and Nicolson, 1972; Robins and Nicolson, 1975). The relative mobilities of antigens on untransformed as compared to transformed fibroblastic cells are different (Edidin and Weiss, 1974). This process of surface antigen modulation was first studied by Old et al. (1968). The effect that modulation of tumour associated antigens may have on the susceptibility of tumour cells to e.g. complement dependent antibody cytolysis is clear since the correct disposition of two IgG molecules is mandatory to fix complement. This is illustrated by the observations of Lesley and Hijman (1974), who showed that removal of about 30 % of the H2 antigens from the surface of S 194 myeloma cells by anti-H2 during a 3-hour incubation at 37° C renders 80 % of the tumour cells refractory to complement lysis. Therefore, transmembrane control of the dynamics of surface antigens may play an important role in determining the susceptibility to immune destruction. Tumour escape may take place by extensive lateral redistribution of antigens into large patches and caps, endocytosis or by antigen shedding (see below).

Many tumour associated antigens may be mobile in the surface of the membrane, capable of cap formation in such a way as described for membrane-bound immunoglobulins on lymphocytes (Leonard, 1973) The amount of antigens and the form in which it is presented may then not trigger the right cells or not trigger them in the right form, resulting in an ineffective immune response: immune serum passively transferred to syngeneic animals grafted with the immunizing tumour fails to modulate the growth of the chemically induced tumour, although the antibodies exhibit lymphocyte- and complement-dependent cytotoxicity for this tumour (Baldwin et al., 1973). Apparently, a phenotype variant of the tumour cells has been selected with antigenic characteristics that makes it resistant against a maybe otherwise effective immune response.

4. Antigen and antigen-antibody complexes

There is a constant synthesis and degradation of cell plasma membranes (Warren and Glick, 1968; Nachbar et al., 1974; Evans and Gurd, 1971) and one of the mechanisms for this membrane turnover which represents also an important mechanism for tumour cells to escape from immune destruction is the shedding of tumour cell associated antigens (Harris et al., 1973). Kapeller et al. (1973) studied the natural release of mouse H-2 antigens by kidney cells and Cone et al., (1971) found that the shedding of surface proteins from both normal and neoplastic cells occurred at a high rate and is dependent on cellular respiration and protein synthesis. This loss of cell surface structures may result from the release of pieces of plasma membrane by the process of pinching off of microvilli or cell surface projections (Nowotny et al., 1974) and/or by shedding of antigens at the molecular level (Alexander, 1974). Neoplastic cells have been characterized by the presence of increased surface proteolytic activity which has been implicated as the perpetual stimulus to divide and thus increase their invasiveness to surrounding normal tissue (Burger, 1973; Nachbar et al., 1974). Currie and Alexander (1974), studying the release of antigen in tissue culture media supernatants of a number of 3-methylcholanthrene induced rat sarcoma cultures concluded that the rate of antigen shedding may actually determine the capacity of a tumour to metastasize. Davey et al. (1976). studying a different type of tumour, reached the same conclusion. Their hypothesis is supported by the findings of Doljanski (1973) and Ben-Sasson et al. (1974) that Rous sarcoma virus transformed cells in culture release surface molecules, that bind specifically to lymphocytes of chickens bearing Rous sarcoma virus induced tumours. Pellis and Kahan (1975) provided more direct evidence for the retained biological activity of tumour antigen released from cultured tumour cells. They showed that animals could be immunized with exhausted tumour cell culture

media, protecting them against a challenge with viable tumour cells. The effects of tumour associated antigens and antigen-antibody complexes on the abrogation of immune destruction of tumour cells has been reviewed by Price and Baldwin (1975) and Baldwin and Robins (1975).

5. *Antibody and the shedding of antigens*

The possible contribution of antibody in inducing shedding of antigen is not known. There is nevertheless considerable evidence that tumour cells *in vivo* are coated with immunoglobulin (Witz, 1973). This has been shown for a variety of tumours including carcinogen induced hepatomas and sarcomas (Witz et al., 1967; Ran et al., 1976; Robins, 1975), spontaneous mammary carcinoma (Ran and Witz, 1970) and polyoma and SV-40 virus induced tumours (Ran et al., 1976; Sobczak and De Vaux Saint Cyr, 1971).

The concept that cell-mediated immunity in the tumour bearing host may be rendered ineffective by such humoral factors was supported by the observations of Hellström and Hellström (1974b) showing that *in vitro* lymphocyte cytotoxicity for tumour cells can be abrogated by serum of tumour bearing animals. Blocking factors in the sera of tumour hosts preventing the specific recognition of tumour cells by immune lymphocytes were shown to be soluble tumour specific immune complexes. The specific as well as non-specific inhibition of effector cell reactivity is due to the interaction of the effector cells with tumour antigen containing moieties (Baldwin and Robins, 1975; Zöller et al., 1976).

E. Conclusions

The multiple host immune defense mechanisms described and the many routes available to make the escape from them

possible makes one wonder how these systems cooperate or counteract one another *in vivo*. One has to realize, however, that many of the different mechanisms described were studied in a variety of systems. The data are clear in the sense that there is an overwhelming evidence that an intact immune response is effective in modulating and even controlling the growth of certain forms of virus induced tumours. The amount of antigens to which the animals are exposed is large i.e., through the expression of these antigens on viral envelopes or on productively infected cells. Where cells are, however, the only source of tumour associated transplantation antigens, as in the case of oncogen induced or spontaneous tumours, no effects of immune suppression on the oncogenesis are detected although during tumour development immune reactions can be monitored and the immune response to these tumours can be modified by tumour antigen-antibody complexes either *in vivo* or *in vitro*.

The lack of detectable immunogenicity of spontaneous tumours even in the absence of an immunosurveillance mechanism raises questions about the importance of the role of immunity in cancer. The fact, however, that the majority of spontaneous tumours are not found to be immunogenic does not imply that they lack such antigens. We have seen abundant evidence for the capability of the immune apparatus to influence tumour growth ranging from restriction to stimulation of tumour growth. It may be that in spontaneous tumour systems these mechanisms are operating at the same time, one balancing the effects of the other and thus preventing the detection of either response (Norbury, 1977). If these two effects can be separated making dissection of the different immune mechanisms feasible, the balance might be tipped towards immunological destruction of the tumour. It will evidently depend upon the etiology of the tumour (spontaneous, chemically induced or virus induced) which of the immune mechanisms are operating. The development of more sensitive immunological techniques will ultimately lead to a better understanding of

the role of immunity in cancer and give a better insight in how the immune response can be made effective in tumour destruction providing the clinician with a hopefully selective tool for the early detection and treatment of cancer.

F. Cellular immune reactions against human bladder cancer cells

Many efforts have been done to show that human tumours expressed cell surface antigens which were characteristic for that histological type of tumour. This was commonly done by showing that lymphocytes of an individual with a given type of tumour were cytotoxic only for tumour cells of that particular tumour type, i.e., that these lymphocytes did not show cytotoxic reactions against histologically unrelated tumour cells. Furthermore, it was shown that lymphocytes from healthy donors did not show cytotoxic reactions against the tumour cells.

Bladder cancer comprises about four per cent of all new cancers. Three times as many men as women develop bladder cancer and the incidence for both sexes is slowly rising.

Circumstantial evidence has implicated a chemical etiology in human transitional carcinoma of the bladder since certain chemicals such as betha-naphthylamine, xenylamine and benzidine, are known to cause bladder cancer.

A host immune response to this type of tumour has been observed (Bubenik et al., 1970). The most striking correlation with both tumour stage and therapy has been reported by O'Toole (1972; 1973a; b; 1974) and can be summarized as follows: patients with transitional cell carcinoma of the urinary bladder were shown to have lymphocytes in their peripheral blood capable of specifically lysing bladder cancer cells *in vitro*.

Several bladder cancer cell lines obtained from individual patients were shown to be susceptible to lysis, whereas

tumour cells derived from other types of cancer were not affected. These observations led to the conclusion that the antigen on the surface of the different bladder cancer cell lines, so called tumour associated antigens, were cross-rereactive. Moreover, the tumour-specific immune response could be observed irrespective of histocompatibility differences between the target cells and effector cells.

Radiotherapy initially induced suppression of the cellular immune response of the patients and could be followed by 1. full restoration suggesting a favourable prognosis; 2. a weak response that appeared associated with early tumour recurrence; or 3. no restoration of the immune response, indicating continued tumour growth after radiotherapy. Successful surgical removal of a localized tumour resulted in a rapid loss of reactivity whereas a positive response was maintained when residual tumours could be demonstrated. The latter observation was explained on the basis of the continuous presence of a small tumour burden.

Cell fractionation studies indicated that the effector cells belonged to a non-T-cell lymphoid cell population.

Similar results were obtained by other investigators (Bean et al., 1974; Bloom et al., 1974; Bloom and Seeger, 1976; Hakala et al., 1974), although the relation between the immune response and tumour stage and therapy was less pronounced.

The purpose of our studies was to investigate the cellular mechanisms of the immune response of bladder cancer patients against their tumour tissue, the degree of specificity of the response and the possible clinical significance of the response. A better understanding of the cytotoxic mechanisms and analysis of the effector cells involved should ultimately lead to an effective modulation of the immune response to cure the tumour bearing individual from his residual tumour. In the following chapters it will become clear that the patient's reaction against the tumour are complex and frequently involve several different types of antigens.

Therefore, it has become an important task to analyse the complexity and to evaluate the significance of the microcytotoxicity assay as a diagnostic or prognostic tool for monitoring the course of the disease.

Cell-mediated cytotoxicity *in vitro* reflects the activities of several different effector cell types and each cell type may involve different effects on tumour growth and they may each operate during different phases of the disease.

Therefore many studies have been directed towards identifying the nature of the cells responsible for the non-specific or natural cytotoxicity against human tumour cells. Various cell types have been implicated in this role including: Fc-receptor bearing (activated T-) cells (Peter et al., 1975; Hersey et al., 1975), mononuclear phagocytic cells (Holterman et al., 1974), null (non-T, non-B) lymphoid cells (Kiuchi and Takasugi, 1976), T-cells (Holterman et al., 1974), and non-T-cells (Bakács et al., 1977).

Many technical variations are employed for the fractionation of cell populations. In chapter IV the identification of a new cell population of T-lymphocytes is described. In Chapters V and VI a number of cell separation procedures are used for the identification of the cells exerting non-specific or natural killer cytotoxicity.

CHAPTER II

CELLULAR MICROCYTOTOXICITY IN A HUMAN BLADDER CANCER SYSTEM: ANALYSIS OF *IN VITRO* LYMPHOCYTE-MEDIATED CYTOTOXICITY AGAINST CULTURED TARGET CELLS

A. Introduction

The recent upsurge in interest in the immune responses to human tumours emphasizes the need for monitoring the specific and nonspecific immune functions. Experimental syngeneic animal studies employing *in vitro* techniques for measuring cell-mediated cytotoxicity for tumour cells demonstrated the presence of neoantigens on tumour cells with specificities identical to those capable of eliciting tumour immune reactions *in vivo* (Hellström and Hellström, 1969; 1974; Baldwin, 1973). These results provided the rationale for measuring the cell-mediated cytotoxicity of human patient lymphocytes against tumour cells. It was assumed that the *in vitro* cytotoxic activity of peripheral blood leukocytes from cancer patients was a manifestation of a state of immunity to tumour-associated antigens (TAA). Many investigators, using mostly the microcytotoxicity test (MCT) (Takasugi and Klein, 1970), have shown that lymphocytes from individual cancer patients preferentially destroy tumour cells of the same histological type rather than normal cells or tumour cells of a different type of tumour, regardless of the allogeneic differences (Hellström et al., 1971; Hellström and Hellström, 1973; Bubenik et al., 1970; 1971; O'Toole et al., 1972; O'Toole et al., 1974; de Vries et al., 1972; Fossati et al., 1971; Fossati et al., 1972; Heppner et al., 1973; Bean et al., 1974).

Bubenik et al. (1970) reported on the presence of TAA on certain cell lines derived from a human urinary bladder car-

cinoma. O'Toole et al. (1972) extended these studies and found a inversed relation between the stage of disease and the cytotoxicity of lymphocytes. The further reported alterations of the anti-tumour immune response with different forms of therapy. Bean et al. (1974) using their modified isotopic MCT (Bean et al., 1973), confirmed the work of Bubenik and O'Toole.

An increasing number of investigators, however, reported their inability to demonstrate "tumour type specificity" (Takasugi et al., 1974; Skurzak et al., 1973; Berkelhammer et al., 1975). However, recently, the occurrence of normal or natural killer cell-mediated cytotoxicity of lymphocytes against tumour cells has been described in healthy animals (Nunn et al., 1973; Greenberg and Playfair, 1974; Kiessling et al., 1975a; Kiessling et al., 1975b; Zarling et al., 1975) and man (healthy donors, HD) (Takasugi et al., 1973; Takasugi et al., 1974; de Vries et al., 1974; Kay and Sinkovics, 1974; Rosenberg et al., 1974; Hersey, 1975a; Hersey et al., 1975b; Heppner et al., 1975; Mukherji et al., 1975b). This effector lymphocyte has been termed the natural killer (NK)-cells.

In the present study, lymphocytes from bladder tumour (BT) and tumour control (TC) patients and healthy donor (HD) were tested in parallel on bladder tumour cells from a cell line (T24) reported to carry TAA (Bubenik et al., 1970; Bubenik et al., 1971; O'Toole et al., 1972), melanoma cells (Mel-I and NKI-4), also reported to carry TAA (de Vries et al., 1972; de Vries and Rümke, 1976), and normal bladder epithelium (HCV). The influence of the selection of target cells and the choice of baseline control on the interpretation of MCT results was analyzed. Disease-related cytotoxicity is defined as statistically significant destruction of disease-related tumour cells by the patient's lymphocytes in comparison with the baseline control. Non-specific cytotoxicity is defined as destruction of a proportion (selective) or all (non-selective) of histologically unrelated target cells by effector cells, while no destruction of any target cell signifies no

cytotoxicity. To investigate whether a disease-related activity of lymphocytes derived from bladder cancer patients, superimposed on the normal cytotoxic effects of HD could be demonstrated (De Vries and Rümke, 1976), the overall effects of lymphocytes from BT, TC patients and HD (tested in parallel) on the different types of cultured target cells were compared statistically.

Although several baseline controls were utilized during this study, the medium control was used as a common baseline for the purpose of comparing the overall cytotoxic effects of the different groups, and the groups were compared statistically by the Wilcoxon test. The value of this analysis is that one can determine the frequency distribution of the normal and/or nonspecific activity of the control donors (HD and TC patients) versus the medium control. Utilization of multiple lymphocyte donor controls and this method of calculation eliminates the possibility of the inadvertant selection of a lymphocyte donor with normal cytotoxicity or growth stimulatory activity for target cells as a control which could result in a conclusion of an apparent disease-related cytotoxicity. Furthermore, it allows one to pool the data from the different experiments, therefore giving an objective answer to the question of whether a disease-related response is superimposed on the normal and/or nonspecific activity of lymphocytes against various target cells.

B. Materials and Methods

1. Bladder cancer patients

Blood specimens were obtained through the cooperation of the clinical investigators of our institutes. A total of 51 patients were tested before (one time) or after (one or more times) therapy. Their ages ranged from 55 - 80 with an aver-

age of 67 years. The clinical staging of the tumour was based on the TNM system of the UICC (1963). The patients were distributed in the various categories as shown in Table I. Bladder tumour patients were tested before therapy, 1 - 3 weeks after radiotherapy and 8 - 10 weeks after cystectomy to reduce the influence of immunosuppressive effects of radiation or surgery. Patients were free of tumour at the time tested after therapy.

Table I

NUMBER AND CLINICAL DISTRIBUTION* OF URINARY BLADDER CANCER PATIENTS

Classification	T-category	no. of patients		
		total	male	female
1. Non-palpable tumour or soft mass				
a) tumour confined to the mucose membrane	T1	17	12	5
b) tumour with infiltration of superficial muscle	T2	16	15	1
2. Palpable tumour with infiltration of deep muscle	T3	18	18	0

* Staging as proposed by the UICC (1963).

2. *Controls*

Lymphocytes from 37 TC patients (20 females; 17 males) were tested, occasionally during but mostly after therapy, with residual tumour present. They ranged in age from 31 - 85 years with an average of 61. A total of 67 HD were tested (37 females; 30 males). The age of HD varied from 17 - 61 years with an average of 31. A summary of control donors of lymphocytes is given in Table II.

Table II

CONTROL LYMPHOCYTE DONORS

diagnosis	abbreviation	no. of individuals	
Healthy donors	HD	67	
Rectal ca.	TC	3	37
Prostate ca.		2	
Mammary ca.		7	
Cervix ca.		8	
Lung ca.		6	
Larynx ca.		3	
Lingual ca.		1	
Stomach ca.		2	
Cerebral ca.		1	
Uterus ca.		2	
Liposarcoma		1	
Penis ca.		1	

All blood specimens were coded by others before delivery. After the tests were performed, a clinical form was obtained describing the diagnosis and clinical characteristics of the patients, the stage and extent of the disease, previous therapy and the planning for future therapy.

3. *Target cells*

Long-term cell lines were used routinely as target cells. Table III summarizes the cell lines used in this series of tests. The cells were trypsinized for serial passage once a week and used in the MCT 6 days after passage, before the cells reached confluency. The medium used for routine tissue culture was Parker 199 + 10 % heat inactivated foetal calf serum (FCS) with 100 IU penicillin and 100 ug streptomycin/ml and freshly added glutamine (0.3 mg/ml). All cultures were checked periodically for the presence of mycoplasma (Vogelenzang and Compeer-Dekker, 1969) and determined to be negative.

Table III

TISSUE CULTURE CELLS USED AS TARGETS IN CYTOTOXICITY ASSAYS

Origin	Cell culture designation	Reference	Source
Transitional cell carcinoma of the urinary bladder	T24	Bubenik et al., 1973	
Metastatic cutaneous melanoma	MEL-1	O'Toole et al., 1974	C. O'Toole
Non-malignant bladder epithelium	HCV-29	J. Fogh (unpublished)	
Cutaneous melanoma	NKI-4	J. de Vries, 1972	J. de Vries

4. Preparation of lymphocytes

Lymphocytes for a single test were obtained from 20 ml defibrinated blood from each donor. All blood samples were processed within 2 hours after collection. Defibrination was performed in 100 ml bottles containing 1 glass bead per 2 ml blood. Leukocytes were obtained by allowing the erythrocytes to sediment through plasma-gel (1 part of plasma-gel (Roger Bellon, Neuilly, France) mixed with 4 parts of blood) in glass tubes with a length of 11 cm and an inner diameter of 23 mm at an angle of 45° for 30 minutes at 37° C. The supernatant was aspirated and centrifuged (10 min, 350 g) and the pellet consisting of erythrocytes, granulocytes and mononuclear cells was resuspended in 1 - 2 ml of the supernatant and placed onto a column of unstained spun nylon with a length of 1 cm and a diameter of 15 mm, which had been prewashed with 8 ml of the supernatant. The nylon column was incubated for 30 min at 37° C. The lymphocytes were eluted with warm medium RPMI-1640 + 5 % FCS to recover the nonadherent cells. The cells were spun down and resuspended in 20 ml isotonic Tris buffered

NH_4Cl, pH 7.2, and incubated for 5 min at 37° C to lyse the erythrocytes (Boyle, 1968). After this incubation, 20 ml of culture medium were added and the lymphocytes were washed 3 times and checked for viability with eosin and trypan blue. Although the cell yield is rather low (about 30 % yield compared to whole blood lymphocytes count), one obtains an almost pure lymphocyte population containing less than 1 % monocytes or granulocytes (Bean et al., 1975). In general, the viability was over 98 %. The preparation of effector cells and target cells is depicted in figure 1.

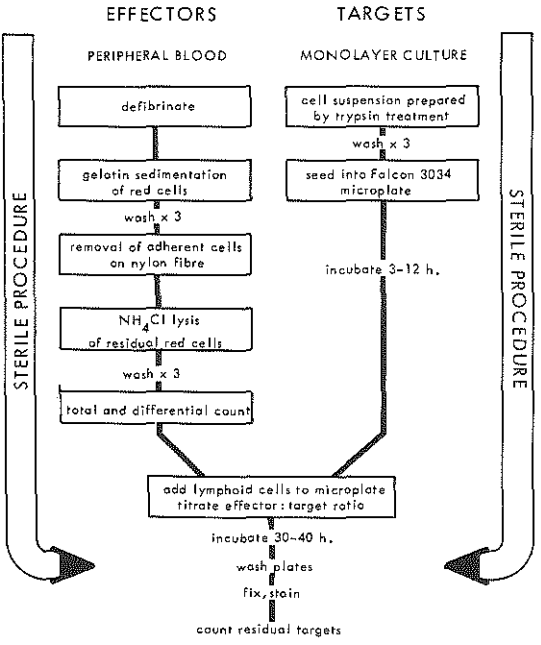


Figure 1. Summary chart of lymphocytes and tumour cell processing.

Table IV

CHECKERBOARD DESIGN OF THE ASSAY

Lymphocyte donors[1]	Target cells: T24 effector/target cell ratio			Mel-I effector/target cell ratio			HCV effector/target cell ratio		
	400 : 1	200 : 1	100 : 1	400 : 1	200 : 1	100 : 1	400 : 1	200 : 1	100 : 1
HD	-56* [2]	-62*	0	18*	16	- 5	36*	13	13
TC	- 7	-38*	0	45*	25*	16*	42*	34*	16
BT-II			- 4			86*			44*
BT-III	99*	84*	45*	100*	88*	96*	36*	24	- 2
BT-I		- 2	-24		26*	14		20*	14

[1] HD = healthy donor; TC = tumour control patients;
BT = bladder cancer patients category I, II and III.

[2] per cent reduction in relation to medium control (MC).
* significant at $p < 0.05$

5. Microcytotoxicity test

This test was based on the method described by Takasugi and Klein (1970). The target cells were collected from monolayer cultures by treatment with 0.05 % trypsin and 0.02 % EDTA (w/v). The cells were washed with medium containing 10 % foetal calf serum and diluted to a concentration giving 50 - 100 per 20 µl medium: 20 µl samples were then plated into wells of microtest plates (Falcon Plastics, Los Angeles, California, USA, no. 3034) by means of a 20 µl micropipet (Hamilton Company, Reno, Nevada, USA). The plates were incubated overnight at 37° C in humidified air + 5 % CO_2. Cell attachment was checked microscopically and, when adequate, the medium was removed and 20 µl lymphocyte samples or medium were added to the wells. The lymphocytes were tested in ratios of 100, 200 and 400 per target cell and each lymphocyte concentration was tested in 8 replicates. After 48 hours of incubation, the lymphocytes were removed by washing the plates twice with phosphate-buffered saline. The remaining adherent cells were fixed and stained with May-Grünwald Giemsa and counted microscopically. The mean number of target cells left in 8 replicate wells after incubation with lymphocytes was calculated. A checkerboard design of the assay demonstrating the minimum number of different types of lymphocyte donors used in each test is given in table IV.

6. Statistical analysis of cytotoxicity testing

For the evaluation of the influence of the choice of baseline control on the interpretation of MCT results, the average number of cells in the wells with lymphocytes derived from patients with carcinoma of the bladder was compared with that found in wells with medium alone (medium control) and control effector cells (HD and TC patients with a tumour of unrelated histology) by the Student t-test. A "p" value of

less than 0.05 was considered significant. The percentage cytotoxicity was calculated by the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{mean number of target cells in control wells} - \text{mean number of target cells with test lymphocytes}}{\text{mean number of target cells in control wells}} \times 100$$

For comparison of the overall effect of different groups of lymphocyte donors the Wilcoxon test was used. A "p" value ≤ 0.05 was considered significant.

C. Results

1. *Influence of the experimental design of the assay on the interpretation of results*

Table V represents one of multiple experiments where the influence of the selection of different types of baselines for specificity control of the interpretation of MCT results becomes apparent. The number of cells remaining in the wells for the different control donors of lymphocytes used as baseline controls (HD (1); TC (2)) differ significantly for both cell lines. Moreover, the cytotoxic effect of one single lymphocyte control donor on the survival of the various types of target cells could be different (HD (2) on T24 and Mel-I against medium control). For instance, patient BT-II shows a disease-related reaction in relation to donor HD (1) and the medium control, but this reaction is considered non-specific if compared with donor HD (2) and donor TC (table V). It is also clear from these data that, again depending on the baseline control selected, the effect of lymphocytes from a control donor on the different cell lines can be reversed. When the cytotoxic effects of the TC patients are calculated with lymphocytes from HD (1) as the baseline, the effects are interpreted as non-specific cytotoxicity against T24 and non-specific stimulation against Mel-I. It is clear, therefore, that no reliable

Table V

ROLE OF BASELINE SELECTION ON THE INTERPRETATION OF CTX

Lymphocyte donor[1]	Target cells: T24 (bladder cancer) no. of cells/well in MC[2]	per cent CTX in relation to MC	HD(1)	HD(2)	TC	Target cells: Mel-1 (melanoma) no. of cells/well in MC	per cent CTX in relation to MC	HD(1)	HD(2)	TC	interpretation of specificity in relation to MC	HD(1)	HD (2)	TC
BT-I	321 ± 47[3]	- 5[4]	2	-57***	-20	103 ± 19	28**	13	32***	39****	non-spec. CTX[5]	Neg.	bladder tumour spec. STIM. and non-spec. CTX	non-spec. CTX
BT-II	150 ± 21	32****	36****	- 2	22**	122 ± 24	15	- 2	20*	27***	bladder tumour spec. CTX	bladder tumour spec. CTX	non-spec. CTX	non-spec. CTX (non-selective)
HD(1)	235 ± 41	7	-	-60****	-22	119 ± 25	17	-	21*	29	Neg.	-	non-spec. STIM. normal CTX	normal STIM. normal CTX
HD(2)	147 ± 24	33****	37****	-	24***	152 ± 29	- 6	-28**	-	9	normal CTX	normal CTX and normal STIM.	-	normal STIM.
TC	193 ± 28	12	18****	31***	-	168 ± 27	-17	-41	-10	-	Neg.	non-spec. CTX non-spec. STIM.	non-spec. STIM.	-
MC	220 ± 35					143 ± 21								

1 HD = healthy donor; TC = tumour control patient; BT = bladder cancer patients, category I and II

2 MC = medium control

3 numbers are means ± S.D. of surviving cells/well remaining attached to the bottom of wells at the end of the test

4 per cent reduction at effector cell:target cell ratio 100 : 1;
* at $p < 0.01 - \leqslant 0.05$;** at $p < 0.005 - \leqslant 0.01$; *** at $p < 0.001 - \leqslant 0.005$; **** at $p \leqslant 0.001$

5 CTX: cytotoxicity; STIM.: growth stimulation; Neg.: no CTX or STIM.

conclusion about specificity can be made, due to the unpredictable effects of lymphocytes of clinical control and healthy donors. Therefore, we used the medium control as the baseline for the calculation of reductions.

The selection of the types of target cells appears to have a similar influence on the interpretation of results. Table VI illustrates the fact that, in this type of assay, the effect of the same BT donor (BT-I) would be interpreted as disease-specific if only cell lines T24 and HCV would have been included in the experiment. When, however, cell lines T24 and Mel-I or all cell lines are included in the experiment, the activity of test lymphocytes which was concluded to be disease-related becomes nonspecific. This confirms the findings of Mukherji et al. (1975b). This could mean that, if more effector cell and/or target cell controls are included in one assay, the probability of finding disease-related cytotoxicity would be greatly diminished.

2. *Nonspecific cytotoxicity of lymphocytes from tumour control patients and natural killer (NK) cell cytotoxicity of healthy donors*

The data in tables VII and VIII clearly show that a proportion of HD and TC patients exert non-specific significant activity against the different target cell lines. We tested 67 HD and 37 TC patients and the results are summarized in Tables VII and VIII. The pattern of reactivity as shown in the tables is representative of the pattern that results from the evaluation of all data. Although some of the lymphocyte preparations of HD and TC patients gave nonselective killing, the majority showed selective reduction of the different target cell lines used in this study. From the HD, 20 per cent showed cytotoxicity against none, 45 per cent against 1, 23 per cent against 2 and 2 per cent against all three target cell lines. For the TC patients, these percentages were respectively 32, 34, 16 and 18 per cent. The normal reactivity,

Table VI

INFLUENCE OF TARGET CELL CONTROL SELECTION ON THE INTERPRETATION OF CTX

Expt. no.	Lymphocyte donors[1]	Target cells: T24 100 : 1[2]	Mel-I 100 : 1	HCV 100 : 1	Interpretation of results in relation to target cells: T24 + Mel	T24 + HCV
23	BT-I	90**** [3]	50**	2	non-specific CTX	bladder tumour specific CTX[4]
	HD	20	15	0		
	TC	44**	14	0		
16	BT-II	90****	0	32***	bladder tumour specific CTX	non-specific CTX
	HD	17	3	29**		
	TC	0	31**	21*		
11	BT-III	87****	72****	5	non-specific CTX	bladder tumour specific CTX
	HD	14	6	43**		
	TC	-13	13	0		

1 HD = healthy donor; TC = tumour control patient; BT = bladder cancer patients, category I, II and III

2 effector cell : target cell ratio

3 per cent reduction in relation to MC.

**** significant at $p < 0.001$; *** significant at $p < 0.005$; ** significant at $p < 0.01$; * significant at $p < 0.05$.

4 CTX = cytotoxicity

Table VII

SELECTIVITY OF NORMAL CYTOTOXIC ACTIVITY OF HEALTHY DONORS

Exp. no.	HD[1] code no.	Target cells: T24 ly/tc[2] 200:1	T24 ly/tc 100:1	Mel ly/tc 200:1	Mel ly/tc 100:1	HCV ly/tc 200:1	HCV ly/tc 100:1
7	(1) 13	12[3]	7	NT[4]	10	52*	41*
8	17	37	40*	-21	18	0	0
	21	47	60*	- 2	20	0	0
9	23	0	0	0	NT	60*	22*
21	81	-10	25	34*	0	22*	17
	82	9	57*	-75*	-25	2	35*
35	159	76*	NT	65*	NT	68*	NT
38	176	-88*	-50*	2	- 7	11	- 9
47	(1)	14	- 6	- 8	-23	29*	8
	(2)	0,9	- 4	- 2	-17	21	21
	(3)	50*	41*	- 2	- 2	-15	-30*
48	(1)	-18	-18	- 5	- 6	- 5	- 1
	(2)	- 4	2	-36*	-11	37*	49*
	(3)	- 5	- 4	4	5	49*	5
	(4)	-39*	-33*	-28*	-24*	-21	- 4
49	(1)	- 9	- 6	2	0	50*	30*
	(2)	11	8	-22	- 7	56*	33*
	(3)	-14	- 3	-14	-23*	43*	25*
	(4)	-12	4	0	5	10	18

1 HD = healthy donor
2 effector cell : target cell ratio
3 per cent reduction in relation to MC. * significant at $p < 0.05$
4 NT = not tested

Table VIII

SELECTIVITY OF NON-SPECIFIC CYTOTOXICITY OF TUMOUR CONTROL PATIENTS

Exp. no.	lymphocyte donor	diagnosis	Target cells: T24 ly/tc[1] 200 : 1	T24 ly/tc 100 : 1	Mel ly/tc 200 : 1	Mel ly/tc 100 : 1	HCV ly/tc 200 : 1	HCV ly/tc 100 : 1
7	15	cervix ca.	24[2]	37*	-33	10	66*	40
15	52	cervix ca.	NT[3]	16	NT	- 2	NT	28*
25	108	cerebral ca.	-33*	15	38*	37*	26*	48*
26	114	stomach ca.	30*	16*	2	NT	22	26
31	139	lung ca.	31*	21	50*	40*	40*	28
37	171	larynx ca.	-39*	-10	-20	-15	8	5
42	195	lung ca.	74*	36*	40*	33*	2	- 4
43	202	mamma ca.	16*	-10	12	10	9	-22
	204	lung ca.	39*	23*	9	7	69*	18
	206	liposarcoma	7	2	6	-29*	4	- 4

1 effector cell : target cell ratio

2 per cent reduction in relation to MC. * significant at $p < 0.05$.

3 NT: not tested

defined as natural killer (NK)-cell activity of lymphocytes, of HD did not appear to be sex- or age-related nor did donors from the laboratory show an increased reactivity over donors from outside the laboratory.

3. *Comparison of the overall cytotoxic effects of lymphocytes from bladder tumour, tumour control patients and healthy donor groups on T24, HCV, Mel-I and NKI-4 cultured target cells*

As shown by our data (Tables V and VI), the number and type of control donors as well as the number of target cell controls greatly influences the chance of determining disease-related tumour reactions, due to the normal reactivity of lymphocytes from HD and the either normal or nonspecific cytotoxicity of TC patients (Tables VII and VIII). Consequently, an impractically large checkerboard design would have to be utilized to clearly demonstrate disease-related cytotoxicity in a single assay. Therefore, we have compared the different groups of lymphocyte donors statistically to investigate whether putative disease-related responses being superimposed on the non-specific or normal cytotoxic responses could be detected, indicating that tumour associated antigens (TAA) are present on cultured tumour cells.

The frequency distribution scatter diagrams illustrate the cytotoxic effects of peripheral blood lymphocytes from the different groups of patients and HD against disease-related target cells and appropriate target cells (figs. 2, 3 and 4). These figures show that there is a wide range in the level of reactivities of lymphocytes from the individuals of each lymphocyte donor group. Before and after therapy, BT patients showed reactivity towards all three target cells used in parallel in each assay; this reactivity was stronger at higher lymphocyte concentrations. The overall cytotoxic effects of lymphocytes from TC patients selected as controls for the determination of specificity of the effector cell

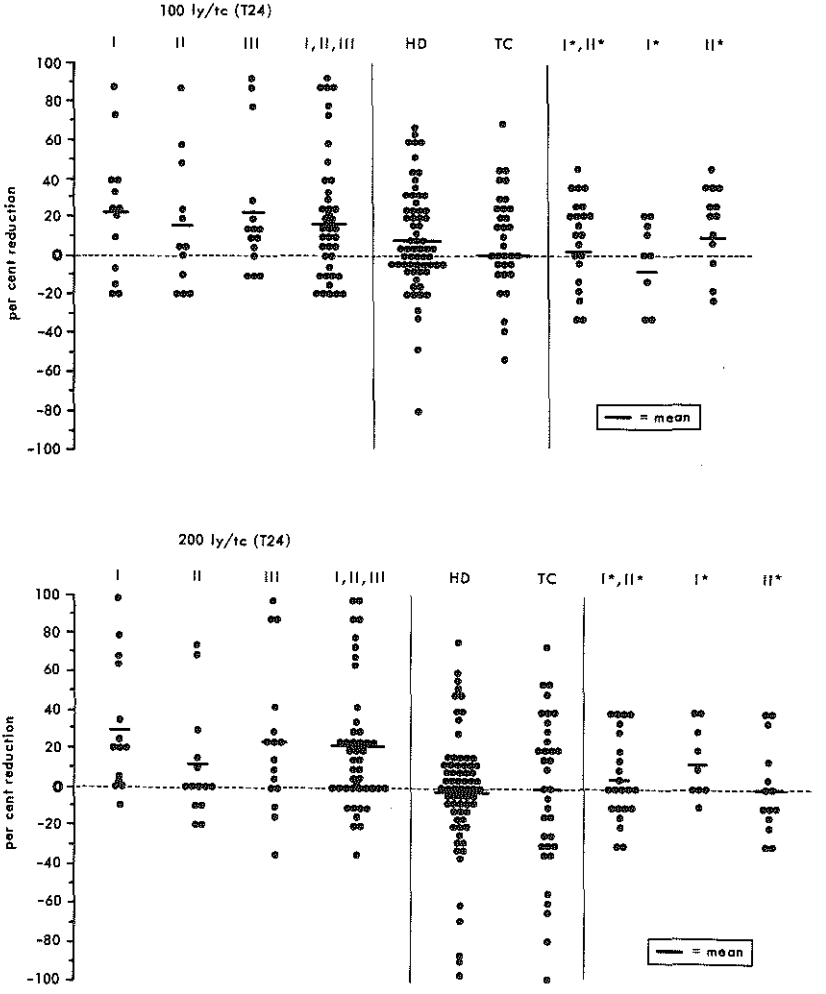


Figure 2. Scatter diagram depicting the cytotoxic effects of lymphocytes from stage I, II, III, pooled I, II, III BT patients before and after* therapy.

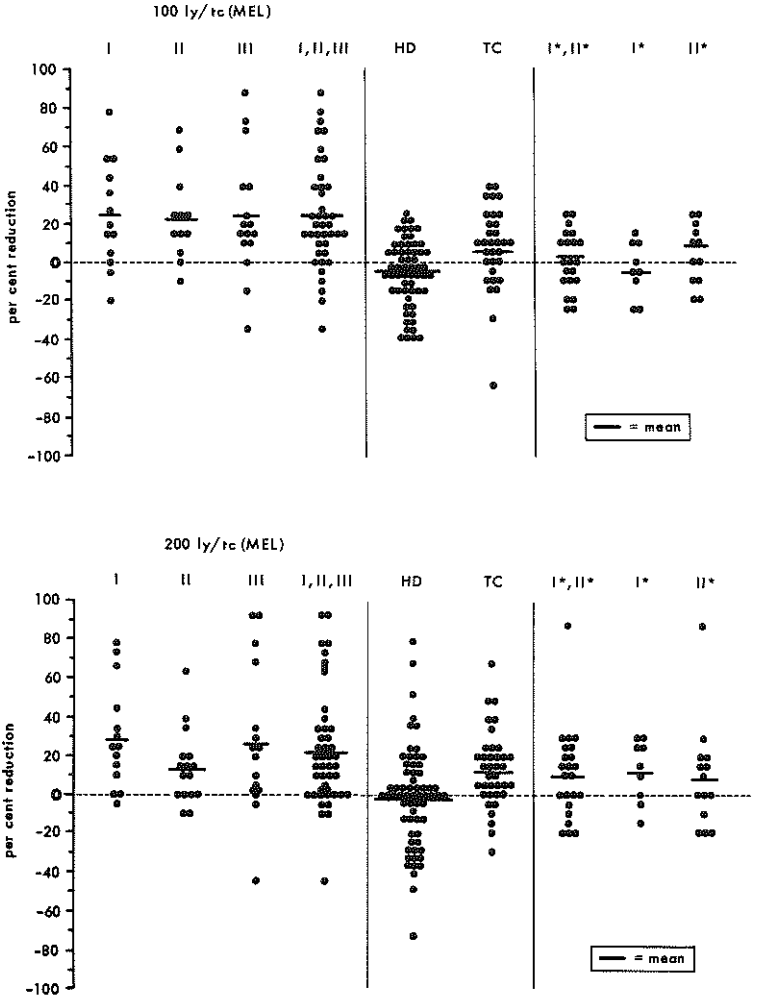


Figure 3. Scatter diagram depicting the cytotoxic effects of lymphocytes from stage I, II, III, pooled I, II, III BT patients before and after* therapy.

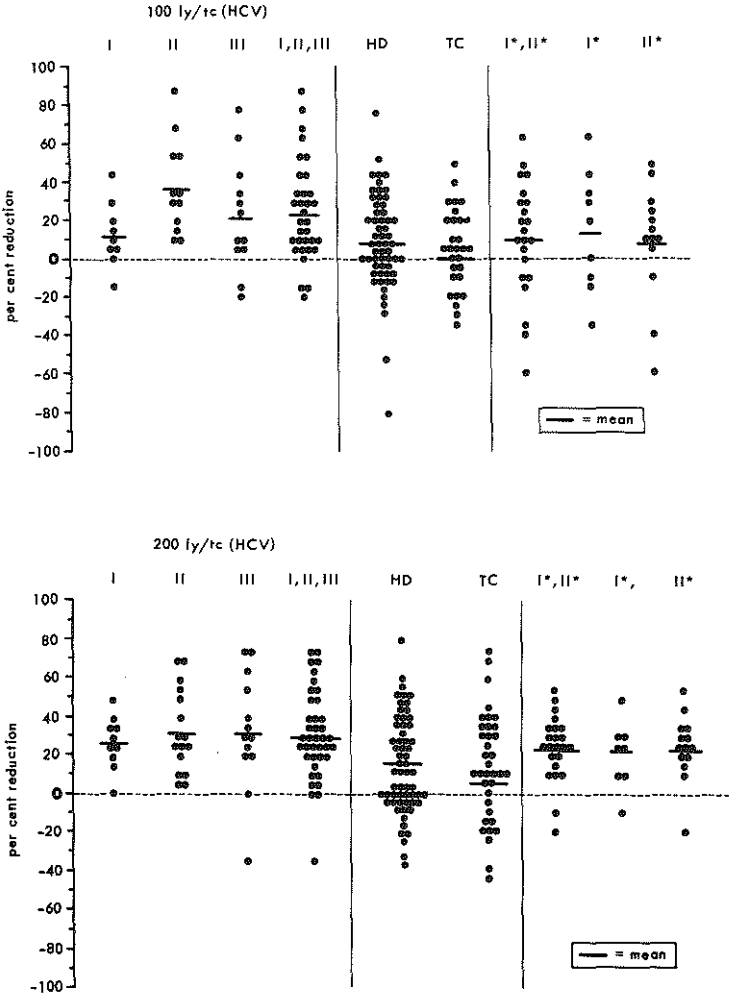


Figure 4. Scatter diagram depicting the cytotoxic effects of lymphocytes from stage I, II, III, pooled I, II, III BT patients before and after* therapy.

level did not differ significantly from those of HD (table IX). It should be emphasized that these patients were tested during or after therapy. When tested within 8 weeks after therapy, the overall cytotoxic effects of lymphocytes from BT patients of all three categories were similar, as was to be expected on the basis of the results shown in table IX (HD versus TC) and hence not significantly different from those of HD and TC patients during or after therapy (Tables IX and X). The overall cytotoxic effects of lymphocytes derived from BT patients of different categories differed from those of HD and TC patients, if the MCT was carried out

Table IX

MEAN PERCENTAGE CTX (± S.D.) OF LYMPHOCYTES FROM BT PATIENTS AFTER THERAPY, TC PATIENTS AND HD ON T24, MEL AND HCV CULTURED CELLS IN RELATION TO THAT OF HD

	T24	p*	MEL	p*	HCV	p*
400 lymphocytes/target cell						
BT stage I	-1 ± 21		8 ± 30		20 ± 18	
BT stage II	23 ± 21		13 ± 21		29 ± 19	
HD	9 ± 38		7 ± 28		27 ± 26	
TC all stages	18 ± 31		22 ± 30		14 ± 33	
200 lymphocytes/target cell						
BT stage I	13 ± 18		11 ± 16		22 ± 18	
BT stage II	0 ± 23		8 ± 27		23 ± 17	
HD	-1 ± 30		-2 ± 26		14 ± 25	
TC all stages	-3 ± 41		12 ± 20		10 ± 27	
100 lymphocytes/target cell						
BT stage I	-9 ± 21		-5 ± 15		23 ± 32	
BT stage II	10 ± 21		9 ± 27		7 ± 30	
HD	8 ± 27		-4 ± 17		8 ± 25	
TC all stages	0 ± 34		6 ± 21		1 ± 26	

Only p values < 0.05 are indicated

Table X

MEAN PERCENTAGE CTX (± S.D.) OF LYMPHOCYTES FROM BT PATIENTS AFTER THERAPY, TC PATIENTS AND HD ON T24, MEL AND HCV CULTURED CELLS IN RELATION TO THAT OF TC PATIENTS

	T24	p*	MEL	p*	HCV	p*
400 lymphocytes/target cell						
BT stage I	-1 ± 21		8 ± 30		20 ± 18	
BT stage II	23 ± 21		13 ± 21		29 ± 19	
BT stage I + II	16 ± 23		12 ± 23		26 ± 18	
TC all stages	18 ± 31		22 ± 30		14 ± 33	
200 lymphocytes/target cell						
BT stage I	-13 ± 18		11 ± 16		22 ± 18	
BT stage II	0 ± 23		8 ± 27		23 ± 17	
BT stage I + II	5 ± 22		9 ± 22		22 ± 17	
TC all stages	-3 ± 41		12 ± 20		10 ± 27	
100 lymphocytes/target cell						
BT stage I	-9 ± 21		-5 ± 15		23 ± 32	
BT stage II	10 ± 21		9 ± 27		7 ± 30	
BT stage I + II	2 ± 23		3 ± 24		10 ± 30	
TC all stages	0 ± 34		6 ± 21		1 ± 26	

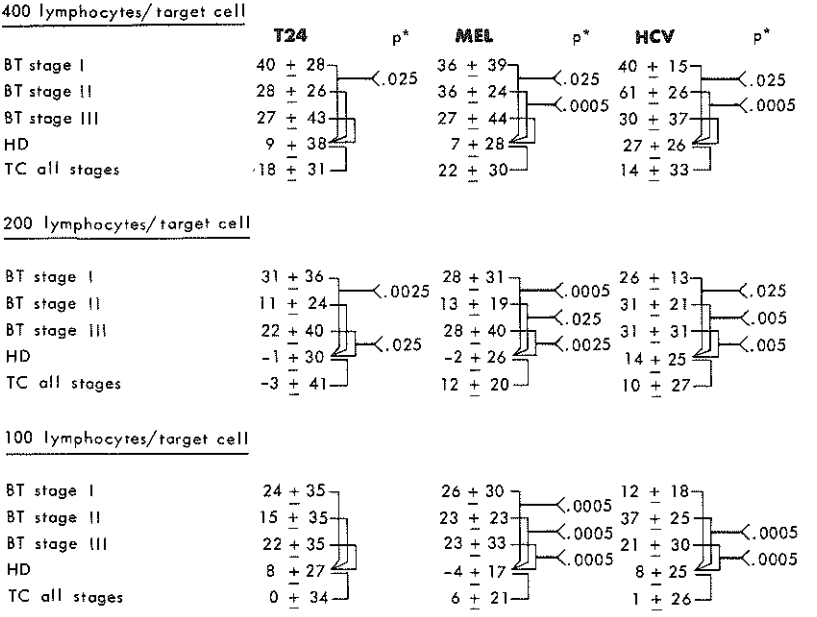
Only p values < 0.05 are indicated

before therapy (tables XI and XII). However, these reactivities were not disease-specific. For example, the lymphocytes of stage-I BT patients were significantly cytotoxic to T24 target cells only in comparison with TC patients, if tested at concentration of 400 : 1. When compared with HD, this apparent specificity is lost, since they are found to be significantly cytotoxic against Mel-I and HCV target cells as well (tables XI and XII).

In general, lymphocytes of BT patients when tested before therapy appeared to be more cytotoxic against the non-disease

Table XI

MEAN PERCENTAGE CTX (± S.D.) OF LYMPHOCYTES FROM BT PATIENTS BEFORE THERAPY, TC PATIENTS AND HD ON T24, MEL AND HCV CULTURED CELLS IN RELATION TO THAT OF HD

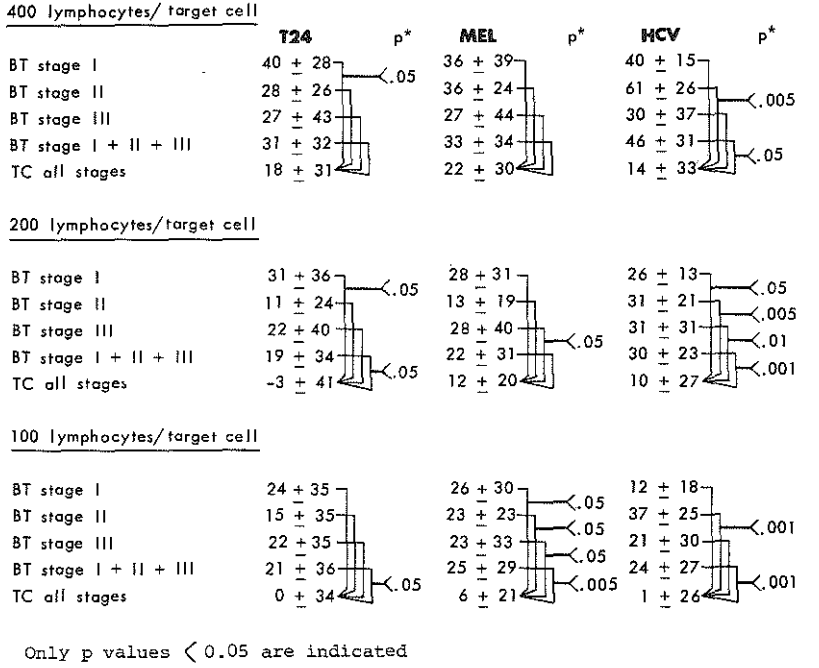


Only p values < 0.05 are indicated

related target cell lines (tables XI and XII) and hence the cytotoxic reactions are concluded not to be disease-related. This contrasts with the conclusion reached by de Vries et al. (1976) on comparing the cytotoxic effects of lymphocytes from melanoma patients with those of HD, using NKI-4 melanoma cells and T24 bladder tumour cells as target cells. We therefore studied the effects of BT patients on these cell lines before therapy. The data are presented in table XIII. Although some patients (BT-I (a and b) and BT-III (b); table XIII) show strong cytotoxic effects against T24 and Mel-I

Table XII

MEAN PERCENTAGE CTX (± S.D.) OF LYMPHOCYTES FROM BT PATIENTS BEFORE THERAPY, TC PATIENTS AND HD ON T24, MEL AND HCV CULTURED CELLS IN RELATION TO THAT OF TC PATIENTS



Only p values < 0.05 are indicated

cells, these effects appear to be even stronger against NKI-4. The percentage CTX of the lymphocytes from the remaining three patients (BT-I (c) and BT-III (a and c); table XIII) is also the highest on NKI-4. From these data, it is clear that NKI-4 melanoma cells are even more susceptible to the cytotoxic effects of lymphocytes from BT patients than T24 and Mel-I. This indicates that the cytotoxic effects of lymphocytes from BT patients are significantly different from the effects of lymphocytes from HD, also when NKI-4 cells are used as target cells.

Table XIII

CYTOTOXICITY OF LYMPHOCYTES FROM BT PATIENTS ON T24, MEL-I AND NKI-4 CELLS

		mean number of cells per well			per cent reduction		
Patient	ly/tc	T24	Mel-I	NKI-4	T24	Mel-I	NKI-4
BT-I(a)[1]	200 : 1[2]	21.3 ± 13.4[3]	23.5 ± 4.9	2.3 ± 1.7	64 ± 5****[4]	32 ± 3**	93 ± 1****
	100 : 1	47.5 ∓ 5.3	25.5 ∓ 7.3	8.3 ∓ 2.8	20 ∓ 5	26 ∓ 3*	76 ∓ 1****
	MC[5]	59.1 ∓ 16.1	34.5 ∓ 8.5	34.3 ∓ 5.0			
BT-I(b)	200 : 1	33.1 ± 6.8	12.5 ± 1.9	3.1 ± 1.6	68 ± 3****	66 ± 1****	95 ± 1****
	100 : 1	71.3 ∓ 23.8	23.3 ∓ 4.7	13.9 ∓ 9.6	31 ∓ 9**	37 ∓ 2**	78 ∓ 4****
	MC	103 ∓ 17	36.8 ∓ 5.5	64.5 ∓ 12.4			
BT-I(c)	200 : 1	29.0 ± 6.4	13.1 ± 2.9	17.9 ± 5.1	1 ± 3	23 ± 2*	58 ± 2*
	100 : 1	31.6 ∓ 8.9	18.1 ∓ 5.6	28.3 ∓ 5.7	- 8 ∓ 4	- 6 ∓ 2	34 ∓ 2*
	MC	29.4 ∓ 5.0	17.1 ∓ 4.0	43.1 ∓ 4.0			
BT-III (a)	200 : 1	45.4 ± 12.1	32.3 ± 9.5	6.0 ± 2.5	23 ± 7*	2 ± 4	83 ± 1****
	100 : 1	48.5 ∓ 15.0	33.0 ∓ 4.2	14.8 ∓ 11.9	18 ∓ 8	0 ∓ 3	57 ∓ 4****
	MC	58.8 ∓ 18.3	33.0 ∓ 8.2	34.6 ∓ 5.3			
BT-III(b)	200 : 1	34.4 ± 5.4	25.1 ± 6.7	8.1 ± 5.0	42 ± 4***	24 ± 3*	77 ± 2****
	100 : 1	49.8 ∓ 14.1	28.4 ∓ 8.8	16.4 ∓ 6.4	15 ∓ 7	14 ∓ 4	53 ∓ 2
	MC	58.8 ∓ 18.3	33.0 ∓ 8.2	34.6 ∓ 5.3			
BT-III(c)	200 : 1	153 ± 18	54.1 ± 18.5	35.0 ± 5.1	-11 ± 11	2 ± 8	30 ± 3**
	100 : 1	149 ∓ 23	49.1 ∓ 8.0	45.6 ∓ 11.6	- 8 ∓ 12	11 ∓ 6	10 ∓ 6
	MC	138 ∓ 22	55.1 ∓ 15.4	50.0 ∓ 11.8			

1 BT: bladder cancer patients, categories I and III, tested before therapy. a, b and c represent 3 individual patients for each category

2 effector cell : target cell ratio

3 numbers are means ± S.D. of surviving cells/well remaining attached to the bottom of wells at the end of the test.

4 per cent reduction in relation to MC. **** significant at $p < 0.001$; *** significant at $p < 0.005$; ** significant at $p < 0.01$; * significant at $p < 0.05$.

5 MC: medium control.

These results again indicate that determination of specificity is totally dependent on the number of controls at both the effector cell and target cell level.

D. Conclusions

In contrast to the well standardized serological methods used routinely in experimental tumour biology, the variables which influence cellular MCT results are only poorly understood, despite the efforts made to analyse the reasons for the discrepant results obtained by different researchers (Baldwin, 1975; Herberman and Oldham, 1975).

Variables greatly influencing the MCT results are the number and the selection of the various types of target cells (table VI, Mukherji et al., 1975a, b; Oldham et al., 1975). In this study only long-term cultured target cells were used to determine whether disease-related cytotoxic reactions by lymphocytes from bladder cancer patients could be detected. The use of established cell lines as a source of target cells is subject to a number of criticisms: 1. they do not resemble the original heterogeneous population of tumour cells *in situ* due to the selection of specific cells which are rapidly dividing; 2. the observation that only a fraction of the attempts to establish cell lines are successful suggests a selection for those types of cells that adapt more readily to *in vitro* culturing. This may lead to changes in the expression of antigens on the membrane of these cells; 3. there are a number of reports demonstrating a greater susceptibility of long-term cultured target cells to destruction by lymphoid cells resulting in an increase in the normal or natural killer cell reactivity of lymphocytes from control donors, although others did not find such differences (Mukherji et al., 1975a, b; Heppner et al., 1975; de Vries et al., 1974; Oldham et al., 1975; Takasugi et al., 1973; Takasugi et al., 1974).

It has been argued, therefore, that the use of established cell lines is one of the factors explaining the inability of several investigators to detect disease-related antitumour reactions (Oldham et al., 1973; de Vries et al., 1974; de Vries et al., 1975; Heppner et al., 1975; Takasugi et al., 1973; Takasugi et al., 1974). Short-term cultured target cells would be less subject to the above criticism and are reported to be less vulnerable to the killing effects of the lymphoid effector cells of HD (Mukherji et al., 1975b; Heppner et al., 1975); in addition, a number of investigators have demonstrated disease-related cell-mediated cytotoxicity in human tumour systems employing short-term cultured target cells (Hellström et al., 1971; Bubenik et al., 1970a; Bubenik et al., 1970b; Fossati et al., 1971; Fossati et al., 1972; Baldwin et al., 1973). However, other investigators found long- and short-term target cells of different histologic origin to be equally affected by normal lymphoid effector cells from HD (Oldham et al., 1975; Takasugi et al., 1973; 1974). The use of short-term target cells involves several problems: 1) they are composed of malignant and nonmalignant cells. This greatly complicates the discrimination between nonspecific, normal and disease-related cytotoxicity, since both malignant and nonmalignant cells may serve as target cells, while they cannot always be distinguished morphologically; 2) they are not readily available for cytotoxicity testing, which is a prerequisite for large scale testing of lymphocytes for clinical investigations. For these reasons and on the basis of the observations mentioned above, long-term cultured target cells were used as target cells in this study.

A second factor which proved to be a crucial variable in the determination of the level of cytotoxicity and the specificity of the antitumour reactions is the choice of the type of baseline control for the calculations. Unpredictable effects of control lymphocytes from HD and TC patients can be seen (tables VII and VIII). Similar observations have been reported by other researchers (Mukherji et al., 1975a; 1975b;

Oldham et al., 1975; Takasugi et al., 1973). Oldham et al., (1975) used the "least active normal donor" in a single assay as the baseline control; however, as has been shown (table V), one individual donor may exert opposite effects on different types of cell lines (Mukherji et al., 1975b) and this would mean that, for each cell line, a different "least active normal donor" should be selected. Moreover, the selection of the "least active normal donor" in one particular assay is dependent on the number of normal donors included in that test and hence makes it incumbent on the investigator to include a large number of normal donors in each test. Repeated use of the same lymphocyte donor whose lymphocytes are proven to be unreactive at one occasion is unreliable, since there is a large fluctuation in the cytotoxic effects of the lymphocytes of one individual donor if tested repeatedly at different time intervals (chapter II, Heppner et al., 1975; Pierce and Devald, 1975; Oldham et al., 1973; Oldham et al., 1975). The repeated use of cryopreserved unreactive lymphocytes is also unreliable (chapter III). Therefore, the medium control was used as a common baseline in this study. Although this type of baseline does not control for the growth stimulating or cytotoxic effects of control lymphocytes from HD and/or TC patients, it is this feature that allows an objective comparison of the overall cytotoxic effects of lymphocytes from the different donor groups in order to investigate whether disease-specific effects of lymphocytes from BT patients are superimposed on the observed normal and nonspecific reactivities. For this purpose, the lymphocytes were always tested on the same set of target cells to reduce the influence of fluctuations in susceptibility to cell lysis by lymphoid effector cells for one particular target cell throughout prolonged culturing (chapter III). Since the patients themselves represent a major variable, the BT patients were divided into three categories on the basis of tumour stage (table I) to maintain each group as homogeneous as possible.

The results obtained indicate that lymphocytes of BT pa-

tients tested before therapy are significantly more reactive against the different types of target cells as compared to those of HD and TC patients (tested during or shortly after therapy) at various lymphocyte : target cell ratios (tables XI and XII). When, however, the same BT patients were tested after therapy, their overall cytotoxic effects against the same set of target cells were similar to those of HD or TC patients (tables IX and X). From these data, it can be concluded that the stronger reactivity is not tumour-specific. This is in contrast to the conclusion drawn by de Vries and Rümke (1976). These authors compared the overall cytotoxic effects of lymphocytes from HD and melanoma patients using T24 bladder and NKI-4 melanoma cells as target cells and found the cytotoxic effect of lymphocytes from melanoma patients to be significantly stronger in comparison to that of HD. Such a difference in cytotoxic effect was not detected on T24 target cells. Therefore, it was concluded that a tumour-related response was superimposed on the nonspecific cytotoxic effects of lymphocytes from melanoma patients if the effects were compared with those of lymphocytes from HD. Table XI shows the frequency of cytotoxic reactions of BT patients against T24 target cells to be smaller as compared to that on Mel-I cells; moreover, the NKI-4 cells appeared to be even more susceptible to the cytolytic activity of lymphocytes from BT patients when their lymphocytes were tested before therapy (table XIII).

It is noteworthy that the three cell lines used throughout this study showed no statistically significant difference in susceptibility to cell lysis by lymphocytes from HD (data not shown).

This latter observation confirms the conclusion that the cytotoxic effects of lymphocytes from BT patients, and possibly of other groups of cancer patients tested before therapy, are significantly stronger against tumour cells, if compared with HD and TC patients during or shortly after therapy. The cytolytic effects were also observed on HCV cultured cells

which are of nonmalignant origin. This susceptibility to cell lysis was also observed by de Vries (personal communication).

It is unlikely that the lack of specificity must be attributed to the lytic activity of monocytes which are reported to give nonspecific cytotoxic effects (O'Toole et al., 1973; Riethmüller et al., 1975), since these cells are present only in low numbers in our lymphocyte preparations. Moreover, the procedure by which effector lymphocytes are isolated seems not to account for the nonspecific reaction observed, since the isolation procedures yield reversed effects on the different types of target cells (Mukherji et al., 1975b; Oldham et al., 1975). Several studies have indicated various subpopulations of lymphoid cells to be responsible for the non-specific and NK-cell reactivity of lymphocytes of HD and TC patients, including C3/Fc-receptor carrying cells, Fc-positive (T-)cells (chapters V and VI; Hersey et al., 1975b; Jondal and Pross, 1975; O'Toole et al., 1973; O'Toole et al., 1974). These different candidates for the cytotoxic effects observed might be present in different concentrations in the peripheral blood of individual donors or in general in different donor groups. The cytotoxicity observed for a single cancer patient may therefore be the result of a combination of disease-related, non-specific and normal or natural killer cell reactivities of the effector cell subpopulations. Since cytotoxicity is merely defined as stronger cytolytic reactivity by test lymphocytes over control, e.g. control lymphocytes or medium control, this complicates the evaluation of disease-related cytotoxic reactions. The observation of increased overall non-specific cytotoxicity of lymphocytes from BT patients before therapy might be due to the presence of cross-reactive embryonic antigens present on tumour cells as described in well-defined animal tumour models (Baldwin et al., 1973; Baldwin and Embleton, 1974).

The discrimination among the different cytotoxic effects will depend on the possibility of separating normal, non-specific or NK- and disease-related reactive effector cells

from each other, assuming there is no overlap in these reactivities. This, however, does not mean only the development of refined cell separation techniques but also modification and more rigorous standardization of the MCT and molecular characterization of the cell surface antigens. We have found that effector cells of the same individual donor have different effects on long term cultured target cells from a particular cell line if these target cells are from different passages. The effector cells used were obtained from one batch of frozen cells and tested in the same experiment on target cells from different passage numbers (chapter III). The fluctuations in the results are probably due to the complexities inherent in the present methodology.

Finally, few studies have been performed on the necessity of histocompatibility between effector cells and target cells as suggested by Doherty et al. (1976) in virus studies. This would make testing of autologous lymphocytes on tumour cells mandatory. The *in vitro* sensitization of lymphocytes from HLA identical sibs are from HLA-matched donors might offer an alternative approach.

CHAPTER III

CRITICAL EVALUATION OF THE MICROCYTOTOXICITY TEST (MCT): ATTEMPTS TO STANDARDIZE THE MCT

A. Introduction

In the previous chapter it was shown that lymphocytes from healthy donors may exert strong normal or natural cytotoxic effects against tumour cells and that the level of the cytotoxic effect was donor dependent. It was for that particular reason that the medium-control was used as the baseline for the calculation of the per cent cytotoxicity. To further study the nature of the NK-cells (see Chapters V and VI) it is of importance to establish whether a standardized MCT measures the cytotoxic capacity of the NK-cells in a reproducible and quantitative fashion. It already has been shown that fresh lymphocytes from the same individual, taken at time intervals and tested in the MCT in separate experiments, show fluctuations in their cytotoxic effect and also that with serial passage of cell lines the target cells change in their susceptibility to lysis by lymphocytes (Levy, 1973; Oldham et al., 1973; Heppner et al., 1975). The observed day-to-day variations seriously compromised the monitoring of the cytotoxic capacity of donor lymphocytes in longitudinal studies. These variations might be reduced by using cryopreserved lymphocytes and target cells. This would allow for the collection of lymphocyte samples from one individual over a long time period followed by testing of these samples or of samples of a number of donors in a single experiment. The variation in this single experiment would then result only from variations in the patients' material together with those due to the freezing procedure of these individual sam-

ples. Furthermore, the cryopreserving technique would then allow to repeat the testing of:

a) aliquots of a large sample of lymphocytes from one individual, providing a standard base-line for evaluation of the level of cytotoxicity of patients' lymphocytes in independent experiments, and
b) lymphocytes isolated from blood taken at time intervals of individual donors on a pool of target cells with known susceptibility to lysis and antigenic content, which then both could be used in different experiments.

The use of a standard base-line and aliquots of one pool of target cells would also allow one to compute the assay variation and the variation in the cytotoxic capacity of the various subpopulations of lymphoid cells.

The successful cryopreservation of a number of functional properties of lymphocytes such as mitogenic stimulation, for histo-compatibility testing and the preservation of a number of cell surface markers (Weiner et al., 1973; Eysvoogel et al., 1973; Strong et al., 1975; Birkeland, 1975) provided the basis for testing the effect of cryopreservation on the cytolytic capacity of lymphocytes. We have asked ourselves the following questions:

1. Is there an effect of the freezing procedure on the cytotoxic capacity of lymphocytes and on target cell lysibility?
2. Is there an effect of serial subculturing of the target cells on the lysibility of these cells by lymphocytes?
3. Does a maximally standardized MCT i.e., using cryopreserved effector and target cells, yield reproducible results when several microcytotoxicity experiments are performed with time intervals.

B. Materials and methods

1. The microcytotoxicity test

The lymphocyte preparations were tested for their cytotoxic capacity in the MCT as described before (chapter II). Target cells were plated 3 hours before addition of the effector cells since pilot studies had shown that this procedure considerably reduced the variation in plating efficiency.

2. Testing of the influence of the cryopreservation procedure on the cytolytic effect of lymphocytes and on the lysibility of target cells and the effect of serial passage of target cells on their lysibility.

The Experimental design is shown in table I.

Plating of the effector cells in the microtest plate.

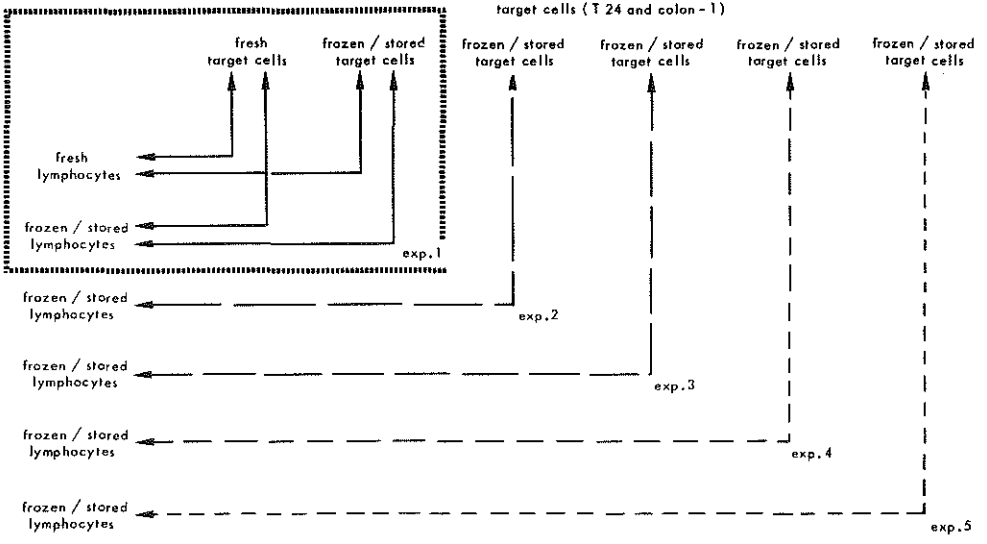
Target cells: T24, a bladder cancer cell line (see chapter II) and Colon-1, were used as target cells. Culturing and harvesting of the cells was performed as described in chapter II (Materials and methods). Cryopreserved target cells were cultured for 1 week before they were used as targets in the MCT.

Lymphocyte isolation

Lymphocytes from peripheral blood, anticoagulated with EDTA, (Merck, Darmstadt, Germany) were obtained by gradient centrifugation using the Isopaque-Ficoll technique (Böyum, 1968). Blood was diluted 1 : 1 with Hanks' Balanced Salt Solution (BSS), layered over the gradient and centrifuged for 13 min at 1400 g. Cells at the interface were aspirated and washed twice with RPMI-1640 medium supplemented with 5 % foetal calf serum.

Table I

EXPERIMENTAL DESIGN



3. Programmed freezing of lymphocytes

Cells were suspended in RPMI-1640 medium supplemented with 20 % foetal calf serum and 10 % dimethylsulfoxide (DMSO) in freezing tubes (2 ml ampoules Greiner 238 S). The DMSO was added within 15 min before freezing of the cells. The lymphocyte samples were frozen at present cooling rates by use of a Cryson BV-4 liquid nitrogen controlled freezer. The cell suspensions were cooled at a rate of -1° C/min to -20° C and then rapidly cooled to -120° C by rapid addition of liquid nitrogen (freezing rate 6° C/min). Vials were then transferred to a liquid nitrogen storage tank where they were kept in the vapour phase until use. Cell suspensions were thawed by rapidly transferring individual tubes from the liquid nitrogen tank into a stream of warm water (approximately 40° C) and agitating the tubes until no ice was present. Subsequently, RPMI-1640 with 20 % foetal calf serum was added stepwise up to 10-fold dilution of the frozen suspension and the cells were washed twice with the medium with 10 % foetal calf serum.

4. Medium

Parker 199 medium supplemented with 10 % foetal calf serum was used. For this study one large batch of medium was prepared, aliquoted and used throughout the experiments so that variations in the results could not be ascribed to variations in the reagents.

5. Statistical analysis of the microcytotoxicity test data

The experiments described in this chapter were designed in a factorial fashion. The data were treated pertaining to as for a completely randomized block design, although with

the effector/target cell ratio variable a split level was introduced. This may have led to an underestimate of the effector/target cell ratio effect because the residual error variance, used as the denominator for the calculation of the Fischer F-value, is characteristically larger than the error variance, associated with the within donor effect.

C. Results

1. Effects of freezing and thawing on the killing capacity of effector lymphocytes and on the susceptibility for lysis of the target cells

The analysis of variance of the data presented in II and III are given in Table IV.

The fresh and cryopreserved lymphocytes were tested in fresh and cryopreserved target cells in a reciprocally way (Materials and methods, table I). The data of the MCT are given in tables II and III and the analysis of variance of these data are presented in table IV.

a) the lymphocytes of the various individual donors tested in this experiment display a significantly different lytic effect against the target cells, confirming the data discussed in chapter II. The donor effect interacts significantly with the other variables studied, viz. D x R; D x L; D x T and D x Q interactions (table IV) and hence the factors are statistically not independent.

b) The effector/target cell ratio, being significant *per se*, yields a significant interaction with the type of target cell (R x Q; table IV) besides the D x R interaction mentioned earlier.

c) The mean lytic capacity of fresh lymphocytes is significantly larger than that of cryopreserved lymphocytes. Inter-

TABLE II

INFLUENCE OF THE CRYOPRESERVATION OF LYMPHOCYTES AND TARGET CELLS ON THE CYTOTOXIC CAPACITY OF LYMPHOCYTES AND THE LYSIBILITY OF TARGET CELLS

donor number	effector/ target cell ratio	per cent reduction of T24 target cells*			
		fresh lymphocytes		frozen lymphocytes	
		fresh	frozen	fresh	frozen
250	200 : 1	53	23	73	39
	100 : 1	29	18	50	39
251	200 : 1	10	-17	2	0
	100 : 1	19	8	21	- 3
253	200 : 1	4	10	-15	8
	100 : 1	3	6	4	0
259	200 : 1	0	-16	11	-13
	100 : 1	0	- 3	20	9
260	200 : 1	6	- 9	4	0
	100 : 1	0	5	-19	- 3
261	200 : 1	9	- 2	1	1
	100 : 1	2	- 9	10	-11

* passage number of T24: 88.

estingly no interaction of the cryopreservation of lymphocytes with that of the target cells were observed, indicating that these effects represent two independent variables (L x T interaction; table IV).

d) The susceptibility to lysis of the two target cells increases as a result of cryopreservation (T effect).

e) The most significant variable in this experiment appeared to be the type of cell used as a target in the MCT. Therefore, the reproducibility of the MCT was separately analysed for each cell line (see below).

Table III

INFLUENCE OF THE CRYOPRESERVATION OF LYMPHOCYTES AND TARGET CELLS ON THE CYTOTOXIC CAPACITY OF LYMPHOCYTES AND LYSIBILITY OF TARGET CELLS

donor number	effector/ target cell ratio	per cent reduction of Colon-1 target cells*			
		fresh		frozen	
		lymphocytes			
		fresh	frozen	fresh	frozen
250	200 : 1	33	25	60	28
	100 : 1	20	15	46	21
251	200 : 1	47	15	79	47
	100 : 1	38	19	75	47
258	200 : 1	43	72	61	79
	100 : 1	39	51	33	40
259	200 : 1	10	39	40	53
	100 : 1	19	34	31	44
260	200 : 1	33	22	61	47
	100 : 1	21	8	41	26
261	200 : 1	- 4	29	34	39
	100 : 1	7	29	12	21

* passage number of Colon-1: 19

2. Effect of subculturing of target cells on the lysibility of the cells

It was of interest to investigate whether the susceptibility to lysis of target cells varied with subculturing of these target cells. Therefore, aliquots of cryopreserved lymphocytes from individual donors were tested on serially passaged target cells in order to minimize variations in the assay due to variations at the level of the effector cells. The results are presented in tables V and VI, and they show that the per cent cytotoxicity fluctuates considerably. The analysis of variance reveals a significant effect of passage number. The effect of passage number of the target cells proba-

TABLE IV

ANALYSIS OF VARIANCE OF THE INFLUENCE OF THE CRYOPRESERVATION OF LYMPHOCYTES AND TARGET CELLS ON THE CYTOTOXIC CAPACITY OF LYMPHOCYTES AND LYSIBILITY OF TARGET CELLS

Effect	Degrees of freedom	Sum of squares	Variance estimate	F-value
D	5	61732.3	12346.5	30.54**
R	1	5073.8	id.	12.55**
L	1	6780.6	id.	16.77**
T	1	15859.5	id.	39.24**
Q	1	170885.3	id.	422.76**
D x R	5	10287.7	2057.5	5.09**
D x L	5	23521.5	4704.3	11.64**
D x Q	5	83130.5	16626.1	41.13**
D x T	5	15640.7	3128.1	7.74**
R x L	1	140.1	id.	0.35
R x T	1	1150.5	id.	2.85
R x Q	1	6176.7	id.	15.28**
L x T	1	75.0	id.	0.19
L x Q	1	1604.3	id.	3.97*
T x Q	1	6521.7	id.	16.13**
Error	672	271632.0	404.2	

*: denotes a probability between 1 and 5 % that the H_0 is valid
**: probability of less than 1 %.
D: donor effect
R: effector/target cell ratio effect
L: fresh or cryopreservation effect of lymphocytes
T: fresh or cryopreservation effect of target cells
Q: target cell type effect

bly account for the significant interaction with the effector/target cell ratio (R x P interaction; table VI). However, an alternative explanation cannot be excluded i.e., that the fluctuations are the result of the lack of reproducibility of the MCT.

Table V

EFFECT OF SERIAL PASSAGE IN VITRO OF TARGET CELLS ON THE LYSIBILITY OF THE TARGET CELLS

donor number	effector/ target cell ratio*	per cent reduction of fresh T24 target cells passage number 88	89	90	91	92
250	200 : 1	23	3	- 7		13
	100 : 1	18	15	-21		2
251	200 : 1	-17	1	5		
	100 : 1	8	17	- 1		
258	200 : 1	10	8	-29	-19	- 1
	100 : 1	6	21	-20	-12	2
259	200 : 1	-16	- 7	5	-10	2
	100 : 1	- 3	- 3	5	-15	0
260	200 : 1	- 9	3	-22	-12	9
	100 : 1	5	- 6	- 5	12	7
261	200 : 1	- 2	-15	-13	-27	18
	100 : 1	- 9	1	-10	- 8	6

* frozen-stored lymphocytes

ANALYSIS OF VARIANCE **

Effect	Degrees of freedom	Sum of squares	Variance estimate	F-value
D	3	1106	368.7	0.90
R	1	3852.8	3852.8	9.40***
E	3	8150.1	2716.7	6.63***
D x R	3	771.3	257.1	0.63
D x E	9	19805.1	2200.6	5.63***
R x E	3	1117.4	372.5	0.91
D x R x E	9	27077.4	3008.6	7.34***
Error	224	91812.9	409.8	

** Only the complete data from donors 258 through 261 were used.

D: donor effect
R: ratio effect
E: experiment effect

***: probability of less than 1 %

Table VI

EFFECT OF SERIAL PASSAGE IN VITRO OF TARGET CELLS ON THE LYSIBILITY OF THE TARGET CELLS

donor number	effector*/ target cell ratio	per cent reduction of Colon-1 target cells passage number 18	19	20	21	22
250	200 : 1	- 5	25	14	25	
	100 : 1	37	15	- 6	15	
251	200 : 1	-67	15	9	17	
	100 : 1	-58	19	16	6	
258	200 : 1	32	72	7	0	58
	100 : 1	31	51	48	6	33
259	200 : 1	22	39	15	-73	38
	100 : 1	12	34	35	26	26
260	200 : 1	12	22	34	6	28
	100 : 1	12	8	44	- 9	39
261	200 : 1	33	29	16	1	33
	100 : 1	13	29	21	25	26

* frozen-stored lymphocytes

ANALYSIS OF VARIANCE**

Effect	Degrees of freedom	Sum of squares	Variance estimate	F-value
D	3	10207.4	3402.5	1.88
R	1	3227.7	3227.7	1.79
P	3	59256.1	19752.0	10.93***
D x R	3	8270.8	2756.9	1.53
D x P	9	26253.5	2917.1	1.61
R x P	3	19772.5	6590.8	3.64*
D x R x P	9	28267.5	3140.8	1.74
Error	224	406565.5	1807.0	

** Only the complete data from donors 258 through 261 were used.

D : donor effect
R : ratio effect
P : passage effect
* : denotes a probability between 1 and 5 % that the H_0 is valid
***: probability of less than 1 %.

3. Analysis of the reproducibility of the microcytotoxicity test

For this purpose two types of experiments were performed. a) Aliquots of frozen-stored target cells from different passage numbers were tested in a single experiment (table VII). b) Target cells with the same passage number were tested at weekly time intervals using aliquots of frozen-stored lymphocytes of individual donors as effector cells in order to eliminate the variables at the level of the effector cells. The

Table VII

INFLUENCE OF PASSAGE NUMBER OF THE TARGET CELL ON THE LYSIBILITY OF THE TARGET CELLS AND THE REPRODUCIBILITY OF THE MCT

		per cent reduction of T24 target cells						
					Experiment number			
donor number	effector*/ target cell ratio	1			2	3	4	5
		passage number						
		61	84	88	88	88	88	88
250	200 : 1	-26	7	-11	39	-18	-13	
	100 : 1	-30	0	- 8	39	- 2	-16	
251	200 : 1	-35			-17	-52	-17	
	100 : 1	-26			8	-23	- 9	
258	200 : 1	16	10	- 6	8	0	-23	-10
	100 : 1	1	15	2	0	- 2	-17	- 8
259	200 : 1	-21	- 6	2	-13	-41	-16	-18
	100 : 1	-28	-14	0	9	-12	-12	- 4
260	200 : 1	-14	-24	1	0	-40	- 5	9
	100 : 1	-27	- 6	9	- 3	2	5	9
261	200 : 1	2	- 9	- 8	1	13	-30	-15
	100 : 1	- 6	- 1	2	-11	- 1	- 3	7

* frozen-stored lymphocytes

analysis of variance is presented in table VIII A
analysis of variance is presented in table VIII B

data for T24 and Colon-1 cells are presented in tables VII and IX, respectively, and the analysis of variance of the data given in tables VII and IX are presented in tables VIII A, B and IX, respectively. Moreover, the donor and ratio effects described earlier were again significant (D, R effects; table VIII A). It should be mentioned that the significance of the second order interaction complicates the interpretation.

Unfortunately, it must be concluded that there are a number of intrinsic variables of the MCT that cannot be controlled, resulting in large fluctuations ranging from reduction to enhancement of target cell growth and hence influencing the level of cytotoxicity, although always the same combinations of frozen-stored effector and target cells were tested. Table VIII A shows also that the susceptibility of the T-24 target cells with different passage numbers, when tested in a single experiment, does fluctuate significantly (P effect). This can also be seen from the data presented in table VI, and hence indicates that the fluctuations in the lysibility of target cells with different passage numbers tested in different experiments indeed is likely to be due to the subculturing of these target cells and can also be ascribed to the lack of reproducibility of the MCT because, when target cells of the same passage are tested in different experiments, significant fluctuations are observed (see above). Aliquots of target cells of the same passage were tested at weekly intervals to monitor the reproducibility of the MCT. From the analysis of variance (table VIII B) it may be concluded that the MCT is not reproducible since the effect of cytotoxicity testing from week to week is significant (E effect).

D. Conclusions

The aim of this study was a systematic analysis of the effect of cryopreservation on human lymphocytes and target

Table VIII A

ANALYSIS OF VARIANCE** OF THE INFLUENCE OF PASSAGE NUMBER OF OF THE TARGET CELL ON THE LYSIBILITY OF THE TARGET CELL

Effect	Degrees of freedom	Sum of squares	Variance estimate	F-value
D	3	3575.6	1191.8	4.19^{xx}
R	1	2737.9	7300.3	25.66^{xx}
P	2	2047.5	1023.7	3.60^{x}
D x R	3	2146.9	715.6	2.52
D x P	6	14530.5	2421.7	8.51^{xx}
R x P	2	627.5	313.8	1.10
D x R x P	6	4455.4	742.6	2.61^{x}
Error	169	48071.5	284.4	

Table VIII B

ANALYSIS OF VARIANCE** OF THE INFLUENCE OF REPEATED TESTING OF THE SAME PASSAGE NUMBER OF THE TARGET CELL ON THE REPRODUCIBILITY OF THE MCT

Effect	Degrees of freedom	Sum of squares	Variance estimate	F-value
D	3	3128.3	1042.8	1.94
R	1	8590.5	8590.5	16.00***
E	4	14093.3	3523.3	6.56***
D x R	3	915.9	305.3	0.57
D x E	12	8478.2	706.5	1.32
R x E	4	5866.9	1466.7	2.73*
D x R x E	12	7264.6	605.4	1.12
Error	280	150339.3	536.9	

** Only the data from donors 258 through 261 were used.

D : donor effect
R : ratio effect
E : experiment effect
* :denotes a probability between 1 and 5 % that the H_0 is valid
***: probability of less than 1 %.

Table IX

REPRODUCIBILITY OF THE MCT

donor number	effector*/ target cell ratio	per cent reduction of Colon-1 target cells: experiment number 1 (passage number 19)	2 (19)	3 (19)	4 (19)
250	200 : 1	28	31	9	
	100 : 1	21	28	9	
251	200 : 1	46	35	28	
	100 : 1	47	30	44	
258	200 : 1	79	10	32	- 7
	100 : 1	40	33	16	- 7
259	200 : 1	53	- 3	8	20
	100 : 1	44	16	30	13
260	200 : 1	47	31	4	15
	100 : 1	26	24	18	31
261	200 : 1	39	14	10	23
	100 : 1	21	11	27	13

* frozen-stored lymphocytes

ANALYSIS OF VARIANCE**

Effect	Degrees of freedom	Sum of squares	Variance estimate	F-value
D	3	1719.0	573.0	0.39
R	1	125.2	125.2	0.08
E	3	34577.3	11525.8	7.79***
D x R	3	1454.5	484.8	0.33
D x E	9	21302.3	2366.9	1.60
R x E	3	7931.1	2643.7	1.79
D x R x E	9	8539.9	948.9	0.64
Error	224	331629.7	1480.5	

** Only the data from donors 250 through 261 were used.

D : donor effect
R : ratio effect
E : experiment effect
***: probability of less than 1 %.

cells and the reproducibility of the MCT. Under the optimally standardized conditions i.e., using cryopreserved effector and target cells, the level of cytotoxicity observed might not be influenced by day to day variations (see later). Several authors have reported that cryopreserved lymphocytes and target cells gave similar results in a long term cytotoxicity assay as compared to fresh cells. This was demonstrated in a rat model system (Ortaldo et al., 1976) and in a human model system (Jeejeebhoy and Lawler, 1976). In the rat system, however, the target cell tumour associated antigens are well defined and hence the specificity of the reactions are well defined likewise. In the human system, mentioned above, the fresh and cryopreserved lymphocytes and target cells were never tested simultaneously and only a limited number of experiments was performed. This study indicates that cryopreserved lymphocytes are less cytotoxic than fresh lymphocytes, extending and confirming previous observations by others (Pavie-Fischer et al., 1975).

Cryopreserved target cells are more susceptible to lysis than fresh target cells, although the cryopreserved cells after thawing were cultured for 1 week before they were used as target cells in the MCT, viz. an irreversible damage of the target cells is not a plausible explanation since these cells would have died off before testing. The factor displaying the most significant effect is the type of tumour cell used as the target, i.e., a large difference in target cell lysibility is noted (table IV; Q effect). The same is true for the passage numbers of a particular target cells i.e., the susceptibility to lysis of target cells fluctuates with subculturing.

When the cytotoxic effects of different donors are compared by testing them in a single experiment a significant donor effect is observed (table IV and VIII A). Such a donor effect, however, is lost when the donors are repeatedly tested in independent experiments (table VIII B and IX). This confirms our previous conclusion, already mentioned in chap-

ter II, that lymphocytes of normal donors do not provide a proper base-line for the calculation of the cytotoxic capacity of cancer patients lymphocytes due to the fact that in a single experiment different donors appear to exert significantly different cytotoxic effects.

Furthermore, this observation implicates that in order to compare the cytotoxic effects of lymphocyte samples taken from one individual at different times or to compare the cytotoxic capacity of lymphocyte samples taken from different individuals, it is mandatory to test these samples in a single experiment since neither cryopreserved lymphocytes, when tested at different occasions, provide a standard base-line nor do aliquots of target cells of the same passage tested at different occasions show comparable lysibility to the lytic effects of the effector cells.

The most crucial finding is that the MCT cannot be used for longitudinal monitoring of cell mediated cytotoxic capacity of patients in experiments performed at different times (table VIII and IX). Technically, the MCT is complex and there are variables such as experimentator errors, cell counting, pipetting, incubator temperature, humidity and CO_2 concentration (Jewett et al., 1976) and these variations are not due to biological phenomena. One of the major drawbacks in the MCT is the long incubation time required, allowing a number of factors to influence the results, factors that apparently cannot be controlled. The development of an assay that measures the cytotoxic effects of lymphocytes in a much shorter time period, limiting the variances in the test, is required. One approach may be the ^{51}Cr-release cytotoxicity assay. This test allows the monitoring of target cell lysis by lymphocytes after 18 h incubation of effector and target cells.

CHAPTER IV

THE IDENTIFICATION OF A NEW SUBPOPULATION OF T-LYMPHOCYTES

A. Introduction

In the study aiming at the testing of the efficacy of lymphocyte subpopulation isolation procedures in order to test the various subpopulations for NK- and K-cell activity, one of the cell surface markers i.e., the complement receptor, was used for fractionation of lymphocytes. Rosettes were made by using IgM coated sheep red blood cells (E_{sh}) plus complement. No IgG was used for sensitization of the E_{sh} to prevent rosette formation by complexing of the IgG-Fc receptor (IgG-FcR) with IgG antibody coated E_{sh}. As controls for the rosettes formed with the E_{sh}-IgM-complement complex in order to show that rosettes were formed via the complement receptor on the surface of the lymphocytes: a. E_{sh}, b. E_{sh} coated with IgM, and c. E_{sh} plus IgM and inactivated complement were each added to lymphocytes in separate tubes. The observation was made that rosettes were formed in the tubes containing lymphocytes and IgM coated E_{sh}.

Receptors for aggregated IgG and antigen-IgG type antibody complexes have been detected on human T-cells (Yoshida and Anderson, 1972). Moretta et al. (1975) recently demonstrated a new receptor on T-cells which is capable of binding antigen-IgM type antibody complexes after culturing the lymphocytes. Our studies indicate that no culturing of freshly drawn peripheral blood lymphocytes is necessary for the expression of this receptor. Furthermore, we have studied the effect of foetal calf serum concentration of the incubation

medium as well as the effect of controlled freezing and thawing of lymphocytes on the capacity of lymphocytes to form rosettes with EA-IgM.

B. Materials and methods

1. Lymphocyte isolation and culture

Lymphocytes from peripheral blood were obtained by gradient centrifugation using the Isopaque Ficoll technique (Böyum, 1968) as described before (chapter III, Materials and methods).

Overnight incubation of purified lymphocytes: cells were stored at 37° C for 18 h at a concentration of 2 x 10^6/ml by placing the tubes with 2 ml cell suspension in a horizontal position in a 37° C incubator.

2. Preparation of anti-E_{sh}-antiserum and the IgM antibody containing fraction

Rabbits were immunized with stroma of sheep red blood cells (E_{sh}) to raise an antiserum rich in IgM type antibodies as described by Kabat and Mayer (1961). IgM was isolated by DEAE chromatography, starting buffer 0.1 M phosphate, pH 7.5. The linear gradient was made by admixing 0.3 M phosphate buffer to the starting buffer. IgM fractions were tested, pooled, concentrated and subjected to Sephadex G-200 gel filtration. The elution buffer was phosphate buffered saline plus 0.15 M NaCl, pH 7.5. The IgM containing fractions were collected and tested for complement dependent haemolytic activity of E_{sh}. Fractions not containing IgG as determined by immuno-electrophoresis were pooled. Partial cleavage products of IgM pentamers were induced by treating the IgM antibody

fraction with dithioerythritol (DTE) (final concentration 0.1 mM) (Chapuis and Koshland, 1974).

3. Techniques for EA-IgM rosette formation

Blood of sheep was mixed 1 : 1 with Alsever's fluid and kept at 4° C until use. E_{sh} were washed 4 times with phosphate buffered saline (PBS) and resuspended at a concentration of 1 x 10^8/ml in RPMI-1640. The E_{sh} were sensitized with a diluted IgM containing antiserum fraction (1 step beyond the agglutination titre) by incubating the mixture at 37° C on a roller bank. The complex formed will be abbreviated as EA-IgM. For the rosette formation equal volumes (250 µl) of EA-IgM and purified lymphocytes (2 x 10^6/ml) suspended in RPMI-1640 supplemented with 5 % foetal calf serum (unless indicated otherwise) were mixed and spun down for 5 min at 250 g, resuspended and incubated at 37° C for 5 min to disrupt possible E_{sh}-cell rosettes. The percentage RFC was determined in a haemocytometer after supravital staining of the lymphocytes with brilliant cresyl blue (0.1 per cent in PBS).

To investigate whether non-complexed IgM-type antibodies could bind to the IgM-receptor on lymphocytes, lymphocyte suspensions (2 x 10^6/ml) were incubated at 37° C with the IgM containing fraction of the antiserum and subsequently washed twice with RPMI-1640 supplemented with 2.5 % foetal calf serum to remove free IgM. The complex formed will be abbreviated as Ly-IgM. Ly-IgM was then incubated at 37° C or 4° C with an equal volume (250 µl) of a 1 % E_{sh} solution.

For inhibition studies we used purified IgM isolated from sera of patients with macroglobulinaemia. The IgM was a gift from Dr. F.W. Putnam (University of Indiana, Bloomington, Indiana, U.S.A.), who also isolated Fc fragments from IgM. These preparations were made available to us by Dr. F. Gmelig-Meyling (University Hospital, University of Utrecht, The Netherlands). A 0.5 ml volume of purified lymphocytes

(1 x 10^6/ml) were incubated for 2 h at 0° C (on melting ice) with 0.125 mg of the inhibitor followed by incubation with EA-IgM and the percentage of rosettes formed was counted.

4. Lymphocyte marker tests

The simultaneous E and EA-IgM rosette formation

In order to study the nature of the cell binding the EA-IgM complex double marker experiments were performed. Thus, since binding of E_{sh} to lymphocytes is an accepted T-cell marker the ability of lymphocytes to form double rosettes was examined (Wybran et al., 1972).

E_{sh}-rosettes and mixed E_{sh}-EA-IgM rosettes were formed by incubating the lymphocytes at 22° C for 20 min with a mixture of E_{sh} and IgM sensitized fluorescein isothiocyanate-labelled E_{sh} (Möller, 1974) abbreviated as EA^{FITC}-IgM. Uncoated E_{sh} and/or E_{sh} treated with normal rabbit serum were used as controls throughout this study. The double rosette were counted using a Litz-Dialux microscope.

5. Direct immunofluorescence for the identification of T-cells

The technique used has been described previously (Knapp et al., 1973). Anti-T-cell antiserum was prepared from a horse anti-human-thymocyte IgG fraction (Upjohn Comp., Kalamazoo, Michigan, U.S.A.) and made specific for human T-cells by exhaustive absorption with insolubilized normal human serum, ABO erythrocytes, B-cells from chronic lymphatic leukaemia cells and cells of the myeloid lines. It was prepared and supplied to us by Dr. Grê Asma from the Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands (Asma et al., 1977a, b).

C. Results

1. EA-IgM rosettes: frequency, properties and the effects of different types of treatment of lymphocytes on the percentage of EA-IgM-RFC

As can be seen in fig. 1, a considerable number of blood lymphocytes are capable of rosette formation with EA-IgM antibody complexes. However, a wide range of reactivities is observed. This confirms the finding of Gmelig-Meyling et al. (1976).

Table 1 shows the results of two representative experiments where the effect of pre-incubation of lymphocytes for

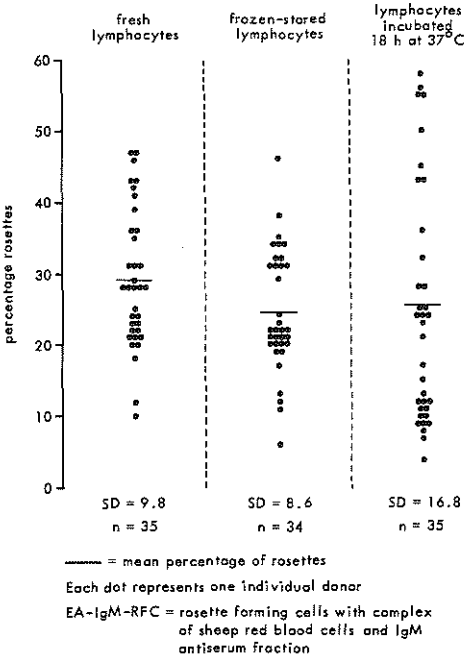


Figure 1: Percentage of EA-IgM-RFC of purified peripheral blood lymphocytes.

18 h at 37° C and programmed freezing and thawing of lymphocytes on their rosette forming capacity of fresh lymphocytes with EA-IgM complexes was studied. Although a decrease or increase in the percentage EA-IgM-RFC after incubation of the lymphocytes for 18 h at 37° C (donors 2, 5 and 7, respectively) and programmed freezing and thawing (donor 1) is seen, the mean percentages of EA-IgM-RFC are approximately the same, regardless of the type of pretreatment (table 1, fig. 1). It was found, however, that pronounced fluctuations in the percentage of rosettes are observed more frequently after 18 h preincubation of lymphocytes at 37° C prior to EA-IgM rosette formation in comparison with fresh lymphocytes than after freezing and thawing of lymphocytes; e.g. after freezing/thawing, the percentage of EA-IgM-RFC was always equal to that of fresh lymphocytes or slightly lower, whereas the percentage of EA-IgM-RFC of lymphocytes after pre-incubation at 37° C was mostly either increased or decreased in comparison to fresh lymphocytes. As can be seen in table I, there is a consistent drop (50 % or more) in the percentage of EA-IgM-RFC after 18 h of incubation of programmed frozen-thawed lymphocytes at 37° C. We have no explanation for the latter phenomenon at present.

It should be emphasized that prolonged incubation of lymphocytes at either 4 or 37° C should be done by placing the tube containing the lymphocyte suspension in a horizontal position (Zeylemaker, personal communication). This results in a 100 % recovery of over 98 % viable lymphocytes (data not shown). Investigation of the effect of different temperatures of incubation and incubation times of lymphocytes with EA-IgM complexes in order to determine the optimal conditions for rosette formation (i.e., 22, 37° C and different incubation times: 0 - 30 min) showed that the procedure described in Materials and methods (this chapter) yielded stable rosettes and reproducible data. No significant difference was found if rosette formation was induced by centrifuging the incubation mixture, supplemented with either 2.5 % or 20 % foetal

Table I

PERCENTAGE OF EA-IgM*-RFC**OF LYMPHOCYTES WITH AND WITHOUT DIFFERENT TYPES OF TREATMENT

	Percentage of EA-IgM*RFC**								
	Experiment 1				Experiment 2				
donor number	1	2	3	4	5	6	7	8	9
fresh lymphocytes	24	40	27	18	20	37	23	41	21
frozen/thawed lymphocytes	12	46	29	16	22	39	20	38	18
lymphocytes pre-incubated at 37°C, 18 h	14	18	23	17	35	29	35	25	NT
frozen/thawed lymphocytes; incubated at 37°C, 18 h	NT	NT	NT	NT	6	13	9	14	0

* EA-IgM = complex of sheep red blood cells with IgM containing antiserum fraction

** RFC = rosette forming cells

NT = not tested

calf serum, at 250 g followed by resuspension and incubation at 37° C for 5 min and incubation of the pellet (20 % foetal calf serum) at 22° C for 20 min prior to resuspension and incubation at 37° C for 5 min (table II). The latter procedure was necessary when double marker experiments were performed for the simultaneous detection of EA^{FITC}-IgM-RFC and E_{sh}-RFC (rosettes of T-cells with E_{sh}) to facilitate E_{sh}-rosette formation.

2. Temperature dependence of EA-IgM-rosettes

Prolonged incubation of EA-IgM-rosettes at 37° C results in a decrease in the percentage of rosettes. After incubation of the rosettes at 37° C for 18 h virtually no rosettes are observed (table III). In order to investigate whether the rosette-forming cell looses its capacity to form EA-IgM rosettes, we repeated the rosetting procedure after incubation of the rosettes for 18 h at 37° C. As can be seen (table III, donors C, D, E and F), the cells are no longer capable of forming rosettes with the EA-IgM. In pilot studies, the ratio of E_{sh} to lymphocytes (50 : 1) used in this test was found to be optimal. Thus a proportion of the EA-IgM might no longer be available for combining with the IgM receptors on the surface of the lymphocytes because the antibody of the EA-IgM has formed a complex with the IgM receptor after dissociation of the (EA-IgM)-lymphocyte complexes or because IgM receptors shed from the surface of the lymphocytes are bound to EA-IgM. Addition of fresh EA-IgM to the rosettes incubated at 37° C for 18 h, however, does not result in rosette formation. Therefore, saturation of the IgM on the E_{sh} by IgM-receptors of lymphocytes seems not a likely explanation for the lack of rosette-formation. When frozen-stored lymphocytes from the same donor were added to the overnight incubated rosettes, rosettes were formed. This indicates that indeed there were still EA-IgM complexes present after incubation for 18 h at

Table II

THE INFLUENCE OF SERUM CONCENTRATION AND TYPE OF INCUBATION FOR ROSETTE FORMATION· ON THE PERCENTAGE OF EA-IgM*-RFC**

Exp. no.	donor number	Percentage of EA-IgM-RFC Rosetting procedure 5 min, 250 g; resuspend 2.5 % foetal calf serum	5 min, 250 g; incubated 20 min, 22° C; resuspend 2.5 % foetal calf serum	5 min, 250 g; resuspend 20 % foetal calf serum
1	4	39	36	38
	5	41	44	42
	6	45	45	42
2	12	24	28	27
	13	34	44	40
3	26	75	78	76
	27	62	75	65
	28	52	57	53

* EA-IgM = complex of sheep red blood cells with IgM containing antiserum fraction

** RFC =rosette forming cells

Table III

TEMPERATURE SENSITIVITY OF EA-IgM* ROSETTES AT 37° C

Percentage of EA-IgM-RFC** at different times after incubation of the rosettes at 37° C

donor number	0 min	10 min	20 min	35 min	2 hour	18 hour	18 hour re-rosetting***	18 h, addition of "fresh" EA-IgM, re-rosetting	18 h, addition of same number frozen lymphocytes of same donor, re-rosetting
A	39	30	26	21	18	11	-	-	-
B	57	33	24	19	-	5	-	-	-
C	30	-	23	24	17	1	1	2	16
D	17	-	-	-	-	0	0	3	9
E	35	-	-	-	-	3	0	0	17
F	20	-	-	-	-	1	0	0	10

* EA-IgM = complex of sheep red blood cells with IgM containing antiserum fraction

** RFC = rosette forming cells

*** re-rosetting = repeat of the EA-IgM rosette procedure (see Materials and methods)

37° C capable of combining with the IgM receptor on lymphocytes and hence IgM receptors were no longer present on the surface of the lymphocytes forming rosettes and incubated for 18 h at 37° C. If one corrects for the fact that twice as many lymphocytes are present after addition of frozen lymphocytes of which 50 % do not form rosettes (table III, donors C, D, E and F), the corrected percentages of EA-IgM-RFC after addition of frozen-stored lymphocytes are the same as before incubation. This could be expected on the basis of data presented in table I which show that programmed freezing of lymphocytes does not significantly alter the percentage of cells capable of forming EA-IgM rosettes.

3. *Inhibition of EA-IgM rosette formation*

To show that indeed the IgM coat of E_{sh} was responsible for the rosette formation, we studied the inhibition of rosette formation by adding human IgM and its Fc-fragments and rabbit IgG as described in Materials and methods (this chapter).

The data shown in table IV indicate that the rosette formation can be inhibited only by IgM or its Fc-fragments. The latter observation indicates that the binding of the receptor to IgM needs only the presence of the Fc part of the IgM molecule.

We therefore investigated whether the IgM antibody of the IgM antiserum fraction could bind to the IgM receptor of lymphocytes, by pre-incubation of lymphocytes with the antiserum and subsequent washing of the incubated lymphocytes to remove excess of free IgM, since free IgG antibodies do not bind to the IgG-FcR *in vitro* (data not shown). The complex to be formed is designated as Ly-IgM. The results are shown in table V and demonstrate that no prior antigen-antibody complex formation is required for binding of IgM type antibodies to the IgM receptor on the lymphocytes. When this experiment was carried out at 37° C the percentage RFC for the lymphocytes

Table IV

ROSETTE FORMATION OF LYMPHOCYTES WITH EA-IgM* AND Ly-IgM** WITH E_{sh}

donors	Percentage EA-IgM-RFC ***		
	EA-IgM + lymphocytes	Ly-IgM + E_{sh}	E_{sh} - RFC ****
KE	27	18	0
RB	18	9	0
PD	17	12*****	0
LD	13	11*****	0
EG	16	19*****	0

* EA-IgM = complex of sheep red blood cells with IgM containing antiserum fraction

** IgM = complex of lymphocytes with IgM containing antiserum fraction

*** RFC = rosette forming cells

**** E_{sh} - RFC = cells forming rosettes with sheep red blood cells

***** Ly-IgM + E_{sh} were incubated at 4° C

from donors KE and RB that results from mixing EA-IgM and lymphocytes is much higher compared to that after mixing Ly-IgM and E_{sh}.

However, as mentioned earlier, EA-IgM-RFC dissociate slowly at 37° C (table III). The same seems to be true for the Ly-IgM complex. The incubation of Ly-IgM with E_{sh} was performed at 4° C for 30 min, since the rosettes were found to be stable at that temperature for a prolonged time (data not shown). Indeed, the percentage of EA-IgM + lymphocytes are similar to that of Ly-IgM + E_{sh} (table V, donors PD, LD, EG).

To determine which subpopulation of lymphocytes formed the EA-IgM rosette, we carried out double marker experiments by incubating lymphocytes with a mixture of fluorescein iso-

Table V

INHIBITION OF EA-IgM* ROSETTE FORMATION BY IgG, IgM AND IgM-Fc FRAGMENTS

Percentage EA-IgM-RFC**

percentage inhibition of rosette formation between brackets

donors	pre-incubation of lymphocytes with medium	pre-incubation of lymphocytes with IgG	pre-incubation of lymphocytes with IgM	pre-incubation of Fc-fragment of IgM
ER	35	32 (8)	8 (77)	9 (74)
WI	35	31 (11)	19 (49)	14 (60)
RE	27	24 (11)	16 (40)	16 (60)
KE	33	30 (9)	11 (70)	12 (64)

* EA-IgM = complex of sheep red blood cells with IgM containing antiserum fraction

**RFC = rosette forming cells

thiocyanate (FITC)-labelled E_{sh}, sensitized with IgM containing antiserum and E_{sh}. After centrifugation, the mixture was incubated for 20 min at 22° C and the medium was supplemented with 20 % foetal calf serum in order to facilitate E-rosette formation, which is used here as a T-cell marker. As shown in table II, there is no influence of the serum percentage and time of incubation used on the percentage of EA-IgM-RFC. Table VI shows the results of the mixed rosetting assay and it can be concluded from these data that the majority of the EA-IgM-RFC (87 - 96 %) are T-cells. One objection that can be made is that the high percentage of mixed rosettes (E_{sh}-rosettes and EA-IgM-rosettes) is due to cross-linkage of IgM bound to

Table VI

SIMULTANEOUS EXPOSURE OF MEMBRANE RECEPTORS FOR IgM AND E_{sh}* BY LYMPHOCYTES

donor number	absolute number of RFC**			percentage EA-IgM-RFC showing E_{sh} binding
	(EA^{FITC}-IgM*** + E_{sh}) RFC	EA^{FITC}-IgM-RFC	E_{sh}-RFC	
1	90	4	1	96
2	45	3	12	94
3	58	9	14	87
4	35	3	27	92

* E_{sh} = sheep red blood cells

** RFC = rosette forming cells

*** EA^{FITC}-IgM = complex of fluorescein isothiocyanate-labelled sheep erythrocytes with IgM containing antiserum fraction

E_{sh}-FITC and E_{sh}. Indeed, it was occasionally observed that the mixed rosettes were not totally disrupted after 5 min incubation at 37° C, a temperature at which the E_{sh}-rosettes are known to dissociate, whereas the EA-IgM rosettes are still stabile (table III). Therefore, we incubated the lymphocytes with a tetramethylrhodamine (TRITC) conjugated anti-human-T-cell antiserum. After washing the lymphocytes to remove excess antiserum, they were mixed with EA-IgM and rosette formation was induced as described. The results shown in table VII lead to the same conclusion: the EA-IgM-RFC belong to the T-cell population of lymphocytes.

4. The dependence of EA-IgM rosette formation on the presence of IgM pentamers

We have mixed the IgM containing fraction of the antiserum with a solution of dithioerythritol (DTE) (final concentration 0.1 mM DTE) to reduce intersubunit disulfides. This results in reduction products, leaving approximately 60 % of the IgM in its pentameric structure (Chapuis and Koshland, 1974). E_{sh} were subsequently coated with the partially DTE cleaved and the noncleaved IgM containing antiserum fraction as described in Materials and methods (this chapter). Thus the percentage of EA-IgM-RFC formed with E_{sh} coated with partially cleaved IgM (with 60 % of the IgM left in a pentameric form) is expected to be approximately the same as the percentage formed with a twofold further dilution of uncleaved IgM antiserum fraction if: a) the dilution of the antiserum is so chosen that the percentage of rosettes formed is dependent upon the antiserum dilution; b) the rosette formation is dependent upon the presence of IgM pentamers.

Therefore, the dilution of the antiserum for the sensitization of E_{sh} was so chosen that the percentage of EA-IgM-RFC decreased about 50 % upon a twofold further dilution of the antiserum.

Table VII

THE IDENTIFICATION OF EA-IgM*-RFC** AS T CELLS WITH ANTI-HUMAN-T-CELL ANTISERUM

Donors	percentage membrane positive lymphocytes with anti-T cell antiserum + percentage T-cells as per cent of total lymphocyte number	percentage membrane positive EA-IgM-RFC and anti-T^+ cells as per cent of total lymphocyte number	percentage EA-IgM-RFC, T^- as per cent of total lymphocyte number	T^+ and EA-IgM-RFC as a per cent of total EA-IgM-RFC number
KR 1	82	41	3	94
RD 2	78	29	0	100
AM 3	86	24	2	92
PD 4	70	17 (17)***	0	100
LD 5	84	13 (13)	1	
EG 6	67	16 (21)	0	100

* EA-IgM = complex of sheep red blood cells with IgM containing antiserum fraction

** RFC = rosette forming cells

*** percentage of EA-IgM-RFC of lymphocytes without prior anti-human T-cell antiserum incubation is indicated between brackets

From the results (table VIII) it can be concluded that the percentage of EA-IgM-RFC indeed decreases 40 % which is expected on the basis of the fact that 40 % of the pentamers are cleaved. This experiment does not entirely exclude the possibility of EA-IgM rosette formation with E_{sh} coated with cleavage products of IgM pentamers, but if such rosettes are formed they are not stable under the conditions employed.

D. Conclusions

The present studies have shown that receptors for IgM and antigen-IgM antibody complexes can be detected on human T-cells. The possibility of a double recognition reaction between receptors for E_{sh} and IgM-Fc is an attractive one but unlikely, however, since the procedure for EA-IgM rosette formation does not favour the E_{sh}-rosette formation (see Materials and methods, this chapter). In fact, in the control tubes incubated in all tests (mixture of lymphocytes and non-sensitized E_{sh}) we have never found rosettes. This binding seems to be mediated by the Fc portion of the IgM antibody as indicated by the inhibition studies with IgM and the Fc fragments of IgM. Our finding that no prior *in vitro* incubation of lymphocytes is necessary for the expression of IgM-Fc receptor disagrees with the results obtained by Moretta et al. (1975) and McConnell and Hurd (1976), but confirm those of Gmelig-Meyling et al. (1976). The difference in results may be due to the fact that we wash the purified lymphocytes at room temperature, which might permit the release of cytophilic IgM antibody that blocked the IgM-FcR. The results of the controlled freezing/thawing experiments indicate that there is no significant change in the percentage of Ea-IgM-RFC. This is not true for lymphocytes incubated at 37^{o} C for 18 h, where we have observed either an increase or decrease in the percentage of RFC. Furthermore, neither the serum con-

Table VIII

DEPENDENCE OF EA-IgM* ROSETTE FORMATION ON THE PRESENCE OF PENTAMERIC IgM TYPE ANTIBODIES

Exp. no.	donor number	EA-IgM-RFC** as percentage of total lymphocytes number: standard rosetting procedure	EA-IgM-RFC** as percentage of total lymphocytes number: pretreatment of IgM-antiserum with DTE*** and rosette formation in the presence 0.1 mM DTE[3]
1[1]	24	17	7
	25	22	11
	26	19	11
	27	34	14
	28	40	24
2[2]	36	27	7
	37	40	23
	38	32	15
	39	28	13
	40	43	32

* EA-IgM = complex of sheep red blood cells with IgM containing antiserum fraction

** RFC = rosette forming cells

*** DTE = dithioerythritol

1 Lymphocytes have been frozen-stored

2 Fresh lymphocytes

3 Pretreatment with DTE of lymphocytes and E_{sh} had no effect on the percentage EA-IgM-RFC

centration of the supporting medium nor the procedures described for incubation of lymphocytes with EA-IgM are found to influence the percentage of rosettes formed.

However, incubation of the EA-IgM rosettes at 37° C leads to dissociation of the rosettes. This is shown to be due to loss of the receptors on the surface of the lymphocytes rather than saturation of Fc portions of the IgM on the EA-IgM complexes by Fc receptors shed from the surface of lymphocytes, as shown by the experiments where rosettes are obtained after addition of frozen-stored lymphocytes from the same donor to the rosettes incubated at 37° C for 18 h. It is shown that the receptors for IgM can be blocked after preincubation of the lymphocyte with human IgM or its Fc fragments, indicating that binding occurs via the Fc portion of the IgM. This is also suggested by the experiments that show rosette formation upon incubation of Ly-IgM (IgM fraction of anti-E_{sh}) with non-sensitized E_{sh}, demonstrating that the receptor for IgM on lymphocytes can bind to non-complexed IgM. Antigen binding through passively adsorbed cytophilic IgM antibody has been reported in chickens (Webb and Cooper, 1973) and mice (Lamon et al., 1975).

The formation of a high percentage of "double rosettes" by lymphocytes upon incubation of lymphocytes with a mixture of E_{sh}- and EA^{FITC}-IgM complexes have demonstrated that the IgM-receptor carrying cells belong to the T-cell subset. This was further illustrated by the immunofluorescence studies which revealed that virtually all cells forming an EA-IgM rosette are also membrane positive after incubation with TRITC-labelled anti-human-T-cell antiserum. Finally, we have demonstrated that the pentameric structure of the IgM-molecule needs to be preserved for binding of the EA-IgM complex to the T-cell IgM receptor.

The exact role of the IgM receptor and of the IgM cytophylic antibodies remains to be established. Recently, Lamon et al. (1975) described an IgM antibody-dependent cell-mediated cytotoxicity against cells of Moloney sarcoma virus infect-

ed mice. They showed that the IgM fraction of antisera could induce normal lymphocytes to kill MSV-infected cells by normal lymphocytes. In an experimental situation, using rabbit IgM sensitized mouse mastocytoma cells, no IgM antibody dependent cytotoxicity was observed (Van Oers et al., submitted for publication).

The IgM antibody induced target cell lysis by lymphocytes may play an important role in tumour immunology. In the sera from congenitally athymic (nu/nu) mice and from normal mice, natural antibodies with specificity for spontaneous and carcinogen-induced tumours have been detected. The antibodies are predominantly of the IgM class (Martin and Martin, 1974; 1975). This suggests a possible tumour rejection mechanism which is thymus independent in terms of antitumour-antibody production and can induce non-sensitized T-cells to become killer cells. Moreover, the natural killer cell activity observed may be IgM and IgG antibody dependent. The verification whether such mechanisms are indeed operational during tumour development may have important bearing for cancer immunology and immunotherapy.

CHAPTER V

CHARACTERIZATION OF NATURAL KILLER (NK)-CELLS AND KILLER (K)-CELLS IN HUMAN BLOOD: DISCRIMINATION BETWEEN NK- AND K-CELL ACTIVITIES

A. Introduction

As we discussed in chapter II, many reports have appeared in the literature demonstrating disease-related anti-tumour cytotoxic activity of lymphocytes of tumour patients (Hellström et al., 1971; Bubenik et al., 1970; O'Toole et al., 1974; Fossati et al., 1972). Documentation of NK cytotoxicity of lymphocytes derived from both normal donors and cancer patients against a wide variety of cell lines seriously questioned the validity of determination of cytotoxicity of tumour patients' lymphocytes (Takasugi et al., 1973; Oldham et al., 1975; Bolhuis, 1977). It is, therefore, evident that in the field of tumour immunology characterization of the nature and specificity of the NK-cell is of paramount importance. Subsequent removal of these cells may improve the chance of detection of disease-related cellular anti-tumour immune reactions in cancer patients.

Antibody dependent and/or antibody independent cell-mediated cytotoxicity may operate in cancer patients. The former effector cells, which are involved in IgG antibody dependent cell-mediated cytotoxicity against IgG-coated mouse mastocytoma cells (K-cell cytotoxicity), bear surface receptors for the Fc portion of IgG (IgG-FcR). Granulocytes and monocytes have also been shown to bear these Fc receptors. A number of cell types have been determined to exert the "spontaneous" cytotoxicity in man, such as IgG-FcR bearing cells (Peter et

al., 1975; Kiuchi and Takasugi, 1976), complement receptor bearing cells (De Vries et al., 1974) or cells carrying both Fc receptors as well as complement receptors (Jondal and Pross, 1975), and activated T-cells (Hersey et al., 1975).

Hence, there are indications in the literature that 1. IgG-FcR are present on both NK-cells cytotoxic against monolayer target cells (Peter et al., 1975; Hersey et al., 1975) or K-562 cells (West et al., 1976) and K-cells (Peter et al., 1975; MacLennan, 1972; Perlmann et al., 1972; Dickler, 1974; Pape et al., 1977), 2. that soluble factors or antibodies (Saal et al., 1977; Akira and Takasugi, 1977) are involved in NK cytotoxicity and 3. that at least a proportion of these cells belong to the T-cell subpopulation of lymphocytes (West et al., 1976, Saal et al., 1977, Biberfield et al.,1975; Kay et al., 1977). The involvement of antibodies, suggested some of these authors, would indicate that the NK cytotoxic cell mechanism is of an ADCC type.

The present study was initiated to answer the following questions:
1) are the cells exerting NK cytotoxicity against the K-562 myeloid cell line, growing in suspension (as measured in a short term ^{51}Chromium release assay), the same as those active against human tumour derived cell lines growing in monolayers (as measured in a longterm cytotoxic assay)?;
2) do the cells derived from different individuals, showing NK- and/or K-cell activity bear the same cell surface characteristics (T-cell markers, FcR, surface immunoglobulins (SIg))?

B. Materials and methods

1. Target cells

P-815-X2, a mouse mastocytoma line, was originally obtained from Dr. R.H. MacDonald, Swiss Institute for Experimental Cancer Research, Lausanne, and supplied to us by Dr.

W. Zeylemaker, Central Laboratory of the Netherlands Red Cross Blood transfusion Service, Amsterdam, and K-562, the myeloid cell line, was obtained from Dr. Grace Cannon, Litton Bionetics, Kensington, Md., USA. The cell lines were maintained in suspension culture in modified MEM supplemented with 10 % foetal calf serum and 0.3 mg/ml glutamine and in RMPI-1640 supplemented with 20 % foetal calf serum and 0.3 mg/ml glutamine without antibiotics.

Colon carcinoma cells and two melanoma lines (Mel-I and NKI-4) were cultured as monolayers. These cells were grown in modified MEM supplemented with 10 % foetal calf serum and 0.3 mg/ml glutamine, trypsinized for serial passage once a week and used in the MCT 6 days after culturing, before the cells reached confluency.

For labelling, 2×10^6 target cells in 0.2 ml medium were incubated with 100 µCi of $Na_2\ ^{51}CrO_4$ solution, specific activity 50 - 400 mCi/mgCr (The Radiochemical Center, Amersham, UK), for 1 h at 37° C and during the last 15 minutes antisera were added to the target cells for ADCC (i.e., P-815 mouse mastocytoma cells). Target cells were washed three times thereafter, the last wash being performed immediately before use in order to minimize the extent of spontaneous ^{51}Cr release.

2. Microcytotoxicity test

The NK-cell cytotoxic capacity was tested using the microcytotoxicity test and was performed as described in chapters II and III. The NK-cell active against monolayer tumour target cells in this system will be designated as NK_{lt}.

3. NK-cytotoxicity and K-cytotoxicity (ADCC)

One tenth ml of ^{51}Cr-labelled IgG sensitized P-815-mastocytoma cells (10^4) and K-562 myeloid cells in Parker 199 + 10 % foetal calf serum buffered with $NaHCO_3$ were added to

round-bottomed small culture tubes (Falcon 2058) together with 0.1 ml of a lymphocyte suspension in RPMI-1640 + 10 % foetal calf serum (0.5×10^6). The tubes were incubated for 4 hours at 37° C in an atmosphere of 5 % CO_2 in air. Each combination was tested in triplicate. Control incubations, in the absence of effector cells always gave less than 15 % spontaneous ^{51}Cr-release. Maximal release in the presence of 5 % saponine was $\geq$95 %. After the 4 h incubation the tubes were centrifuged at 400 g for 5 min and 100 µl of the supernatant was transferred to a counting vial and the radioactivity measured in a Packard gamma spectrometer. The percentage of specific ^{51}Cr-release was calculated as $R = (E-S)/(M-S) \times 100$, where E = counts per minute in the experimental tube, S = spontaneous release and M = maximal release in the presence of saponin.

It has been shown that only lymphocytes exert K-cell activity in the ADCC system when IgG-coated mastocytoma cells are used as target cells (McDonald et al., 1975; Zeylemaker et al., 1975). Monocytes have no lytic effect in this system. The NK-cell active in the ^{51}Cr-release assay against K-562 cells will be designated as NK_{st}.

4. 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.

5. Purification of lymphocyte subpopulations

a. Separation of T- and non-T-cells by E-rosette sedimentation

T-lymphocytes possess receptors for E_{sh} and this was

used to separate these cells. Therefore 5 ml of lymphocytes (4×10^6/ml) in RPMI-1640 were mixed with 10 ml of foetal calf serum and 10 ml of E_{sh} at 1×10^8/ml in RPMI-1640 in disposable Falcon 2070 tubes. The cell suspensions were centrifuged for 30 min at 150 g, and incubated for at least 1 h at 4° C. Three ml of the supernatant was left on the pellet and the cell pellet was carefully resuspended, 8 ml of cold medium was added, and the cell suspension was layered onto 10 ml of Ficoll-Isopaque and centrifuged at 400 x g for 40 min at 22° C. After centrifugation the cells from the E-RFC enriched pellet fraction and E-RFC depleted interface fraction were collected and tested for NK- and K-cell activity, and for the presence of cell surface markers. No ammonium chloride buffer was used for lysis of the red blood cells since this abrogates the NK-cell activity as measured in short-term cytotoxicity assays (Kay et al., 1977).

b. Separation of cells lacking FcR

The technique described by Kedar et al. (1974) with some modifications (Van Oers et al., 1977) was used. Culture flasks (25 sq. cm, Falcon 3013) were treated with 5 ml of a solution of 0.05 mg/ml poly-L-lysine (mol. weight 85,000, Sigma Chemical Co., St. Louis, Mo., USA), for 45 min at 22°C. After repeated washing (2x) with PBS 2 ml of a suspension of E_{sh} (1×10^8/ml) was added to the culture flasks and was allowed to adhere for 30 min at 22° C. After removal of non-adherent cells by repeated washing with PBS (2x) the then confluent monolayer of E_{sh} was lysed with H_2O, and washed three times with PBS. The erythrocyte ghost monolayer was now incubated with 5 ml of a 1 : 64 dilution of rabbit anti-E_{sh} serum (IgG fraction) for 30 min at 37° C and washed with RPMI-1640 5 times thereafter. Four ml of a lymphocyte suspension (10×10^6/ml) in RPMI-1640 + 10 % foetal calf serum was then added to the culture flasks and incubated at 37° C for 10 min. The flasks were centrifuged horizontally in a swing-

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	number of experiments
Ficoll-Isopaque purified lymphocytes	100	100	7
Lymphocytes plus iron and magnetism treatment	80 ± 3.1	80 ± 3.1	10
T-cell fraction (E-RFC enriched fraction)	55 ± 7.0	68 ± 4.8	6
Non T-cell fraction (E-RFC depleted fraction)	7 ± 1.3	8 ± 1.4	6
EA-RFC deplected fraction	63 ± 6.3	64 ± 9.3	12
EA-RFC recovered fraction	21 ± 4.3	23 ± 6.8	11
Unfractionated lymphocytes cultured for 1 week	60 ± 1.9	69 ± 4.5	3
EA-RFC depleted fraction cultured for 1 week	43 ± 4.8	60 ± 5.8	9

* Lymphocyte isolation and fraction separations were performed as described in Materials and methods.

out bucket at 400 g for 5 min and incubated for a further 10 min at 22°C. Subsequently, the nonadherent cells were recovered by collecting the medium covering the monolayer and gently washing the flasks 3 times with medium. For preparation of the EA-RFC depleted fraction this procedure was repeated. The procedure is depicted in figure 1.

6. 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 (Bolhuis, 1977). 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 % foetal calf serum. The column was then incubated for 30 min at 37° C. The lymphocytes were eluted with RPMI-1640 plus 5 % foetal calf serum, and the eluted cells were washed in medium.

7. Preparation of monocyte enriched fraction

Monocytes were prepared from Ficoll-Isopaque purified mononuclear cells. One milliliter leukocyte suspension (30 x 10^6/ml) in RPMI-1640 plus 20 % foetal calf serum was added to a Falcon petri dish (60 x 15 mm) and cells were allowed to adhere to the surface by incubating the cells for 1 h at 37°C. After washing the plates thoroughly to remove non-adherent 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 morphologic criteria and size of the cells, as determined electronically.

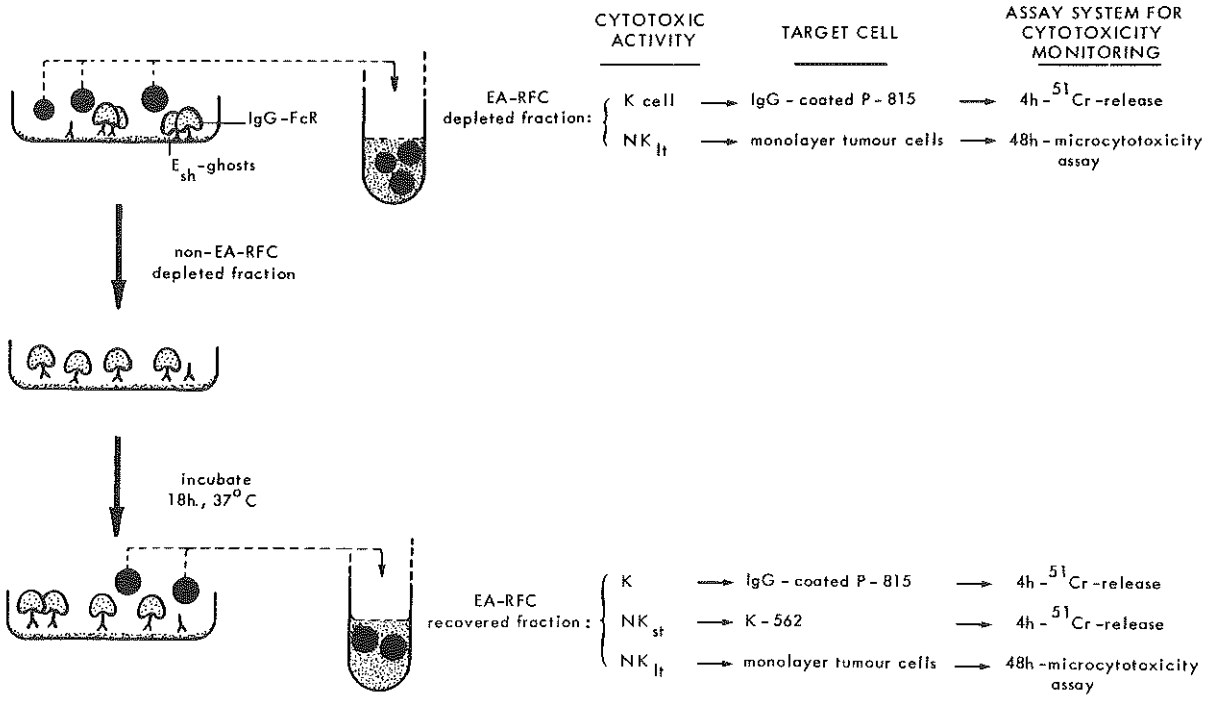


Figure 1. Schematic picture of the separation of EA-RFC depleted and recovered fractions and their cytotoxic activity, their target cell and the cytotoxicity assay used to monitor the cytotoxic activity of the cell populations.

8. E-rosettes (E-RFC)

E_{sh} were stored at 4° C in Alsever's solution and used between 1 and 3 weeks after collection. E_{sh} were treated with neuraminidase by incubating 1 x 10^8 E_{sh}/ml PBS and 2 units/ml of neuraminidase for 30 min at 37° C on a rollerbank (Behringwerke AG, Marburg, Germany). For the test 0.1 ml of a lymphocyte suspension (2 x 10^6/ml) was mixed with 0.1 ml of E_{sh} suspension in foetal calf serum (1 x 10^8 cells/ml). After centrifugation for 5 min at 200 g and incubation for 1 h at 4° C the cell pellet was gently resuspended and the percentage E-RFC scored by counting 200 cells in a haemocytometer after staining the lymphocytes with 1 % brilliant cresyl blue. Cells binding 3 or more E_{sh} were counted as rosettes.

9. EA-rosettes (EA-RFC)

A suspension of washed E_{sh} (1 x 10^8 cells/ml) was incubated with an equal volume of 1 : 32 diluted rabbit anti-E_{sh} serum for 30 min at 37° C on a roller bank. The EA-complexes were washed twice with cold PBS and resuspended in RPMI-1640 (1 x 10^8/ml) and 0.1 ml of the suspension was mixed with an equal volume of a lymphocyte suspension (2 x 10^6/ml), the mixture was then centrifuged at 200 g for 5 min and incubated for 20 min at 22° C. After careful resuspension of the cell pellet the cell mixture was incubated at 37° C for 5 min to disrupt possible E-RFC. The percentage of EA-RFC was determined as described above.

10. Membrane immunofluorescence

This was done as described before (Knapp et al., 1973; Asma et al., 1977). In short, isolated lymphocytes were fixed for 10 minutes in 0.04 % formol solution in PBS and washed

with PBS plus 1 % bovine serum albumin (BSA). Fixed cells were vitally stained by mixing equal volumes of cell suspension (20 µl, 2 x 10^6 cells) and the appropriate dilution of TRITC-conjugated anti-T-cell antiserum (T) and incubation for 30 minutes at 22° C, gently shaking the mixture every 10 minutes. The preparation and specificity testing of the T serum is published elsewhere (Asma et al., 1977). After washing with PBS plus 1 % BSA, the cells were then stained with FITC-labelled goat anti-human Fab (GaHu/Fab, Nordic, Tilburg, The Netherlands). After washing the cells, 20 µl of the stained cells were mixed with 20 µl of buffered glycerol (1 part PBS, pH 7.8, plus 9 parts of redistilled glycerol (Merck)), placed on a cover glass (24 x 32 mm) and covered with an objective glass. The cover glass is then sealed with paraffin.

Two hundred mononuclear cells were identified in phase-contrast and examined for the possession of T-cell specific antigen and SIg employing the two wave-length method (Faulk and Hijmans, 1972). The Leitz-Dialux microscope with phase-contrast (transmitted-light) was equiped with a Ploem illuminator with the following filter combination a) 2 x KP 490, 1 mm GG 455 or 475, 4 mm BG 38 and K 515, KP 560 for FITC excitation and emission respectively, b) K 515, KP 560, 1 mm BG 36 and K 580 for TRITC excitation and emission, respectively.

11. Culturing of lymphocytes

Lymphocytes were cultured for 7 days in RPMI-1640 + 20 % foetal calf serum by placing the culture tubes (Falcon 2058) containing 2 ml lymphocyte suspension (2 x 10^6/ml) in a horizontal position at 37° C in an atmosphere of 5 % CO_2 in air in an incubator (Bolhuis and Nooyen, 1977).

12. Programmed freezing of lymphocytes

The optimal approach for the characterization of the effector cells in experimental models is a combination of cell separation techniques with simultaneous assessment of cell surface markers and reactivity in various assays (Bonnard et al., 1977). This requires large numbers (500 x 10^6) of purified lymphocytes. In order to perform all assays described simultaneously, cryopreserved lymphocytes were used throughout this study.

The cryopreservation was performed as described before (Bolhuis and Nooyen, 1977 and chapter III).

C. Results

1. Presence of FcR on NK- and K-cells

The myeloid cell line K-562 has been shown to be extremely susceptible to natural (or "spontaneous") cytotoxicity (Jondal and Pross, 1975) in a 4 h ^{51}Chromium release assay and indeed unfractionated lymphocytes (fractions A, table II, column NK_{st}) of all normal donors tested in our study exhibited NK-cytotoxicity against this cell line. Furthermore, we employed cells from established cell lines as targets to monitor NK-cell cytotoxicity in an MCT, yielding similar results (fractions A, table II, column NK_{lt}). P-815 coated with IgG was used for monitoring the K-cell activity. It should be noted that all fractions of lymphocytes shown in tables II and III of one individual donor were examined simultaneously in the same experiment for NK-, K-cell activity and the presence of cell surface markers. This ensures optimal information regarding the composition of subpopulations of lymphocytes present in the various effector cell preparations, and the results obtained by simultaneous testing are not subject to the criticism of possible experiment to experiment variations.

TABLE II

PER CENT CYTOTOXICITY OF VARIOUS LYMPHOCYTE FRACTIONS

	per cent reduction of target cells				
		NK_{lt} [1]		NK_{st} [2]	K [3]
lymphocyte donor	NKI-4	Mel-I	colon	K-562	P-815
Donor 313					
A) unfractionated lymphocytes	37*	25*		20*	14*
B) T-cell fraction: E-RFC	15*	- 5		34*	
C) E-RFC depleted fraction	43*	38*		32*	
D) EA-RFC depleted fraction	33*	11		4	0
Donor 314					
A) unfractionated lymphocytes	64*		44*	35*	39*
B) T-cell fraction: E-RFC	15		9	46*	25*
C) E-RFC depleted fraction	55*		33*	35*	19*
D) EA-RFC depleted fraction	18		28	4	5
Donor 316					
A) unfractionated lymphocytes		22*	44*	24*	
B) T-cell fraction: E-RFC		20*	19*	13*	
C) E-RFC depleted fraction		13	40*	24*	
D) EA-RFC depleted fraction	19*			2	0
Donor 317					
A) unfractionated lymphocytes	35*		31*	47*	
B) T-cell fraction: E-RFC	17*		40*	21*	
C) E-RFC depleted fraction	- 9		38*	34*	
D) EA-RFC depleted fraction			35*	0	0
Donor 320					
A) unfractionated lymphocytes	15		48*	14*	23*
D) EA-RFC depleted fraction	- 3		4	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 : K cell cytotoxicity against IgG-coated P-815 (4 h ^{51}Cr-release assay)

* p value ≤ 0.05

TABLE III

COMPOSITION OF THE MONONUCLEAR CELL POPULATIONS PRESENT IN THE ISOLATED FRACTIONS

lymphocyte donor	% E-RFC	immunofluorescence				% EA-RFC
		% T^+	% Ig^+	% T^+/Ig^+	% T^-/Ig^-	
Donor 313						
A) unfractionated lymphocytes	71	73	8	1	18	12
B) T-cell fraction: E-RFC	89	93	1	0	6	7
C) E-RFC depleted fraction	5	16	30	1	54	39
D) EA-RFC depleted fraction	76	89	1	5	5	1
Donor 314						
A) unfractionated lymphocytes	82					22
B) T-cell fraction: E-RFC	95	90	1	0	9	14
C) E-RFC depleted fraction	3	6	30	2	62	61
D) EA-RFC depleted fraction	87	93	1	0	6	0
Donor 316						
A) unfractionated lymphocytes	76					20
B) T-cell fraction: E-RFC	85	96	0	0	4	3
C) E-RFC depleted fraction	3	19	45	1	36	50
D) EA-RFC depleted fraction	93					1
Donor 317						
A) unfractionated lymphocytes	74	80	8	2	10	17
B) T-cell fraction: E-RFC	84	97	2	0	1	3
C) E-RFC depleted fraction	3	17	36	0	47	73
D) EA-RFC depleted fraction	77	98	1	0	1	0
Donor 320						
A) unfractionated lymphocytes	80	87	6	1	6	20
D) EA-RFC depleted fraction	96	96	0	0	4	0

Lymphocytes were not treated. The average yield of B 55 % of A
C 7 % of A
D 43 % of A

As shown in table II (fractions D, column NK_{st}) depletion for EA-RFC resulted in a complete loss of cytotoxicity against the K-562. That the depletion procedure used for EA-RFC was indeed efficient is shown in table III (fractions D, column % EA-RFC). Furthermore, these EA-RFC depleted fractions showed no longer K-cell activity in a cytotoxic test (table II, fractions D, column IgG sensitized P-815). This suggests that the cytotoxic cells bear FcR on their surface (NK_{st} cells). The same fraction of lymphocytes were simultaneously tested for NK_{lt} activity on the established cell lines (NKI-4, Mel-1, Colon), and varying results were obtained. For instance, the EA-RFC depleted lymphocytes from donor 313 (table II, fraction D) were still cytotoxic against NKI-4 melanoma but not against Mel-1 melanoma target cells. The identical fractions of donors 314 and 320 showed no cytotoxicity when tested against NKI-4 and Colon-1 target cells, and the same fraction of donor 317 was cytotoxic against Colon-1. These results uggest that in this long-term MTC assay, with established cell lines as target cells, another subpopulation of cells i.e., cells without FcR on their surface, are also involved in the NK-cell lytic process (NK_{lt}-cells). These observations imply that the NK cytotoxic mechanism of a proportion of the effector cells, is not likely to be of an antibody-dependent type of reaction, since this fraction is virtually devoid of EA-RFC (table III, fractions D, column % EA-RFC), and since the ADCC against IgG-coated P-815 is negative (table II, fractions D, column K-cell cytotoxicity). That FcR bearing cells are also cytotoxic against the monolayer cell lines can be concluded from the observations that the EA-RFC depleted fractions are not cytotoxic, whereas the unfractionated lymphocytes of that donor show cytotoxicity (table II, fractions A and D). That the cell population in fractions A are the most heterogeneous can be concluded from the analysis of cell surface markers (table III, fractions A). From these data it can be concluded that at least two cell populations are showing NK-activity in the different cytotoxic assay systems employed i.e., both IgG-FcR ne-

gative and IgG-FcR bearing cells are cytotoxic against established cell lines as tested in a long-term cytotoxicity assay (NK_{lt} cells), but only FcR bearing cells are cytotoxic against K-562 myeloid leukaemia cells as tested in a short-term cytotoxicity assay (NK_{st} cells).

2. NK- and K-cell activity of T- and non-T-cell fractions

West et al. (1976) recently reported that human E-RFC fractions, obtained under optimal experimental conditions i.e., containing both high-avidity and low-avidity E-RFC (West et al., 1977), showed NK cytotoxicity against the K-562 cell line. The E-RFC depleted fractions, however, were never devoid of NK-cell activity.

We repeated these experiments using both the K-562 cell line and the NKI-4, Mel-1 and colon monolayer cell lines as target cells. Therefore, T-cell fractions (E-RFC cells) and non-T-cell fractions (E-RFC-depleted cells), obtained by rosette formation and separation under optimal conditions were tested for NK cytotoxicity. Both T- and non-T-cell fractions are shown to be almost equally cytotoxic against K-562 target cells, and the extent of reactivity is more or less comparable to the unfractionated lymphocytes (table II, fractions B and C, columns NK_{lt} and NK_{st}). From the cell surface marker analysis of the T-cell fraction (table III fractions B, columns % E-RFC and % T) it can be concluded that the T-cell fractions are extremely pure, i.e. pure lymphocytes containing less than 1 % SIg positive cells, and virtually no cells lacking T-cell specific antigen. Furthermore, it can be concluded that a proportion of T-cells bear FcR (table III, fractions B, column % EA-RFC) and show K-cell activity against IgG sensitized P-815 cells (table II, fractions B, column NK_{st}). The results from the cytotoxicity experiments with the NKI-4, Mel-1 and colon cells as targets are comparable to those with the K-562 cells. Attention should be given

to the fact that T-cells, fractionated by E-rosette formation and subsequent separation of the E_{sh}-RFC on Ficoll-Isopaque are less capable of re-rosetting with E_{sh}. The percentage T^+-cells indicates, however, that this fraction contained pure T-cells. The importance of the latter observation became even more apparent when the non-T-cell fractions were examined. The interpretation of cytotoxicity data obtained with the non-T-cell fractions (Fraction C) is even more complex.

As can be seen from our data (table II, fractions C, column NK cytotoxicity) this fraction is also cytotoxic against all types of target cells used. The efficiency of E-RFC depletion is checked by E-rosette formation of the non-T-cell fraction and this fraction appears to contain virtually no T-cells, i.e. 3 - 5 % (table III, fractions C, column % E-RFC). Analysis of the T-cell markers by means of the anti-T-cell serum, however, indicates that 6 - 17 % of the cells in the non-T-cell fraction can be identified as T-cells. The SIg bearing cells are not likely to contribute to the cytotoxic effects, since the increase of SIg bearing cells from $\pm$ 8 % in population A to $\pm$ 40 % in the interface (table III, fractions C, column % SIg) did not increase the percentage cytotoxicity (table II, fractions C, column NK cytotoxicity). Recent experiments showed that lymphocyte fractions, after depletion of E-RFC and EA-RFC, thereby increasing the percentage of SIg bearing cells up to 80 %, yielded no significant cytotoxicity against K-562 (data not shown).

It is unlikely that monocytes are involved in the NK cytotoxic process against K-562 since a) purified lymphocyte fractions (see Materials and methods, this chapter) are equally or more cytotoxic as compared to the unfractionated cells (Ficoll-Isopaque isolated, without iron treatment of the cells), excluding a direct NK cytotoxic capacity of monocytes; b) fractions of lymphoid cells enriched for monocytes (table IV) show significantly decreased cytotoxicity; c) adding monocytes to the pure lymphocyte fraction does not influence the per cent NK cytotoxicity, suggesting that monocytes do not

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, K-562 AND THE COMPOSITION OF THE MONONUCLEAR CELL POPULATIONS OF THE ISOLATED FRACTIONS

	Target cells			Cell surface markers				
effector cells	NKI-4[1]	Mel-1[1]	K-562[2]	% E-RFC	% T^+	% Ig^+	% T^-/Ig^-	% EA-RFC
Nylon purified lymphocytes	39*	83*	47*					
Nylon purified lymphocytes + 1 % monocytes	49*	78*	50*					
Nylon purified lymphocytes + 5 % monocytes	58*	83*	48*					
monocytes enriched[3] fraction	27*	69*	19*	29	24	9	67	76

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

play an indirect (helper) effect in NK cell killing; d) all lymphocyte fractions were tested in an ADCC against IgG-coated E_{sh} (a monocyte dependent antibody cytolysis assay) and no cytotoxicity was observed. A representative experiment is shown in table IV.

D. Conclusions

In various experiments, using frozen-stored lymphocytes of normal donors, these cells exhibited NK- activity against monolayer cell lines and K-562 cells growing in suspension, and showed K-cell activity (ADCC) activity against IgG sensitized P-815 cells. A proportion of the NK-cells i.e., those showing cytotoxicity against K-562 (NK_{st}-cells), have similar cell surface characteristics as K-cells. One of the characteristics is the presence of IgG-FcR on a proportion of the NK- and on all K-cells as demonstrated by the abrogation of NK-cell and K-cell activity against K-562 and IgG sensitized P-815 cells, respectively, after EA-RFC depletion of the purified lymphocytes by adsorption of the EA-RFC onto IgG-E_{sh} ghost monolayers. This conclusion is confirming other studies involving the characterization of NK- and/or K-cells (Peter et al., 1975; Kiuchi and Takasugi, 1976; Jondal and Pross, 1975; Pape et al., 1977; Pross and Baines, 1976; Kay et al., 1977). Recently, Bakács et al. (1977) showed, that removal of IgG-FcR bearing cells did not result in a complete loss of NK-cell activity. These investigators, however, employed monolayer cell lines as target cells in a longterm cytotoxicity assay. Our cytotoxicity experiments, including both monolayer cultures (NK_{st}-assay) and suspension cultures (NK_{st}-^{51}Cr-release assay) also showed, that still another, IgG-FcR negative, lymphocyte exerted NK-cell activity against exclusively the monolayer cultures (table III, column NK_{lt} cytotoxicity). Our data also show that this NK-cell activity was selective i.e., killed only a proportion of the cell lines, and that both FcR po-

sitive (NK_{st})- and negative (NK_{lt})-cells may have different targets, since the removal of FcR bearing cells sometimes resulted in the loss of cytotoxicity against one, but not against the other type of monolayer target cells. The fractionation of lymphocytes into T- and non-T-subpopulation by means of E-rosette formation and separation, yielding T-cells in the pellet fraction and "non-T-cells" in the interface fraction provided some interesting results. Both T- and non-T cell fractions appeared to be cytotoxic against both monolayer cell lines as well as K-562 cells (our data, West et al., 1976). 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 non-T cell fraction contained virtually no T-cells. When, however, an a/T-cell serum (Asma et al., 1977a, b) was used for the identification of T-cells the following results were obtained: 1) the T-cell fraction contained pure T-cells; 2) the so-called non-T-cell fraction contained a significant percentage of T-cells as shown by the anti T-cell antiserum. That this is not due to non-specific binding of TRITC labelled a/T-cell serum to the cells is illustrated by the two wavelength analysis of SIg bearing cells and T-cell specific antigen bearing cells. The two wavelength method allows the detection of both markers on the membrane of individual lymphocytes. Such analysis revealed that 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, virtually no double staining is observed (table III, column % T^+/Ig^+, fractions A and C). Furthermore, we have not observed positive staining of monocytes, bearing FcR, indicating that the membrane fluorescence is not due to binding of the labelled antisera to IgG-FcR. This argues against the fact that the detection of T-cells with serum is due to nonspecific membrane staining and indicates our observation that T-cells, after rosette formation and separation, do not form E-rosettes as readily as before the E-RFC depletion. This conclusion

is supported by the good correlation between % T-cells as determined by E-rosette formation and by antiserum, when performed before fractionation of these cells on Ficoll. The implications of this finding are important and may explain a number of the apparent contradicting results in the literature. West et al. (1976; 1977) concluded that the T-cells showing the NK-cell activity belong to a subpopulation of T-cells bearing low affinity receptors for 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 appearance of T-cells with low affinity receptors for E_{sh} after this separation procedure in the interface. Since these low-affinity E-RFC have been shown to exhibit the strongest NK-cell activity (West et al., 1976) this would 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 100 % T-cells, see above) at the same lymphocyte target cell ratio, since the latter is relatively depleted and the former relatively enriched for T-cells bearing low-affinity receptors with E_{sh}.

Hersey et al. (1975) showed T-cells, bearing FcR, to be cytotoxic. Kiuchi and Takasugi (1976) defined the NK-cell as a null cell, i.e. without T- and/or B-cell characteristics except the presence of IgG-Fc-receptors. These authors used E-rosette formation and separation and subsequently checked the purity of the separated fractions by E-rosette formation. Our data clearly demonstrate that for testing of the purity of the cell fraction after purification other assays for the characterization of a cell subpopulation should be employed than the one used for the separation of the cell subpopulation i.e., it was demonstrated that not all T-cells form E-rosettes after E-RFC-separation on Ficoll.

Most likely, a proportion of the E-rosettes, apparently the low-avidity E-RFC (West et al., 1977), dissociate during the E-RFC fractionation procedure on Ficoll, and will be found

in the interface i.e., the so-called non-T-cell fraction, and a proportion of these T-cells may bear FcR (Samarut et al., 1976). Thus, 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 the cells is the interface fraction with the a/T-cell antiserum, however, indeed showed the presence of T-cells. West et al. (1976) reported that the E-RFC depleted fractions were seldomly depleted of NK-cell reactivity. Our data are in accordance and the simultaneous analysis of cell surface markers performed strongly suggest that indeed T-cells are present in this E-RFC depleted fraction. The analysis of cell surface markers also showed that pure T-cells (E-RFC, anti-T-cell serum positive) can form EA-rosettes and exert K-cell activity (ADCC) confirming other data (Van Oers et al., 1977; Samarut et al., 1976; Dickler et al., 1974).

Bakács et al. (1977) state on the basis of their experiments that non-T-cells are responsible for NK-cell activity against monolayer cultures. They, however, found that the E-RFC enriched (T-cell enriched) fraction was always cytotoxic, unless passed over a nylon column before cytotoxicity testing. Saksela et al. (1977) recently showed that cells showing "spontaneous" activity were retained on nylon and this may explain the loss of NK-cell activity, which Bakács et al. (1977) observed. Our observations and those of others that B-cell enriched fractions (West et al., 1976; Bakács et al., 1977) and monocyte enriched fractions (our data) had no direct NK-cell activity strengthens the conclusion that the "contaminating" T-cells in the E-RFC depleted (i.e., "non-T-cell" fraction) are responsible for NK-cell cytotoxicity. From the data discussed so far it can be concluded that at least two populations of NK cells exist:

1) NK-cells showing NK cytotoxicity against all types of target cells tested in long-term and short-term cytotoxicity experiments and with IgG-FcR on their cell surface (NK_{lt} and NK_{st}, IgG-FcR positive);

2) NK-cells showing NK cytotoxicity against monolayer cultured target cells tested in MCT, with no demonstrable IgG-FcR on their cell surface as demonstrated with EA-RFC and ADCC.

Recently, the generation of Fc receptors and hence NK_{st}- and K-cell activity after prolonged culturing of EA-RFC depleted cells has been demonstrated (Chapter VI, Ortaldo et al., in the press). One might argue that these cells are generated during the 48-hour incubation period required for the MCT and hence the same type of effector cells was responsible for the NK-cell killing as in the short-term ^{51}Cr release assay using K-562 as target cells. Our observations that EA-RFC depleted cell fractions, cultured for 48 hours did not show any cytotoxicity against T-24 cells in a 4 hour ^{51}Cr-cytotoxicity test, whereas the cells showed cytotoxicity in a 48 h MCT argues against this possibility (data not shown).

K-cell cytotoxicity is known to be antibody-dependent, and the presence of IgG-FcR on NK-cells of antibodies may indicate that the NK cytotoxicity could be of an ADCC type for the following reasons:

1) lymphocytes already have Ig with specificity for certain target cells bound to their surface *in vivo* (Akira and Takasugi, 1977; Kay et al., 1977; Pross and Jondal, 1975);
2) antibody is released by lymphocytes and binds to FcR bearing cells during the cytotoxic assay (Blair and Lane, 1975; Pape et al., 1977).

The first possibility would then explain our observation that EA-RFC negative cells showed NK cytotoxicity against monolayer cultures, since their IgG-FcR were already saturated with IgG. The second type of reaction would require functionally intact FcR on the membrane of lymphocytes.

The question whether the FcR is functionally involved in the NK cytotoxic mechanism will be the subject of chapter VI.

CHAPTER VI

ANALYSIS OF THE INVOLVEMENT OF THE IgG-Fc RECEPTORS ON LYMPHOCYTES IN THE NK-CELL CYTOTOXICITY

A. Introduction

In the previous chapter it was shown that there exist at least three populations NK-cells: 1. a NK_{lt}-cell belonging to a subset of lymphocytes with an IgG-FcR, e.g., exerting NK-cell activity against monolayer target cells, as monitored in a 48 h MCT, and showing antibody dependent lysis of P-815 cells; 2) a NK_{lt}-cell without detectable IgG-FcR, e.g. exerting NK-cell activity against monolayer target cells, as monitored in a 48 h MCT, but not showing antibody dependent lysis of P-815 cells; and 3) a NK_{st}-cell with an IgG-FcR, e.g., exerting NK-cell activity against ^{51}Chromium labelled K-562 cells as monitored in a 4 h isotope release cytotoxicity assay and showing antibody dependent lysis of P-815 cells.

For the NK_{lt}-cell without an FcR on its surface, it is clear that its cytotoxic mechanism is not antibody dependent.

The author contends that the characterization and purification of the effector cells, particularly in human systems, is of crucial importance for a better analysis of their cytotoxic mechanism, the immunologic specificity of their action and their possible role as an immunosurveillance mechanism.

The analysis of cytotoxicity in various combinations of effector cells and target cells has presented evidence that target cells may be lysed not only by lymphocytes, but by a number of cell types (see Introduction, chapter V). Furthermore, it can be concluded from the data presented in chapter V that target cells can be killed by more than one type of lymphocyte. In the past much attention was devoted to the

lysibility of various target cells (chapters II, III and Herberman and Oldham, 1975). With the identification of so many types of effector cells and new subsets of lymphocytes (chapters IV and V) attention should now be given to the phenomenon of discrete "selective susceptibility to lysis" (chapter V) i.e., the lysibility of a particular cell will depend on the number of types of potential effector cells for which it may serve as a target. In the previous chapter it was shown that the various types of NK-cells, FcR positive and negative, are likely to belong to the T-cell subset which also bear low-affinity receptors for E_{sh} (West et al., 1977).

West reported that this low-affinity E_{sh}-RFC are probably pre-T-cells. On the basis of our cell surface marker analysis of the lymphoid cells in each fraction (chapter V) it was concluded that these cells most likely accounted for non-T-, non-B-cells, as defined earlier by Takasugi as null cells (see discussion chapter V). The important question remains whether the FcR for IgG present on the surface of the other two types of NK-cells is functionally involved in the lytic process. The experiments described in this chapter will focus on this question.

B. Materials and methods

The cell isolation, cryopreservation, separation, type classification, isotope labelling techniques and the cytotoxicity assays for monitoring the NK and K-cell activities are described in chapters II, III and V (Materials and methods).

1. Isolation of the FcR bearing cells

All procedures for the separation of cells lacking FcR

were performed as described in Materials and methods in chapter V. The adherent cell fraction was collected by incubating the monolayers plus the adherent cells for 18 h at 37° C and then collecting the medium with the cells that had detached spontaneously.

2. Culturing of lymphocytes

Lymphocytes were cultured for 7 days in RPMI-1640 + 20 % foetal calf serum by placing culture flasks (Costar 3075) containing 10 ml lymphocyte suspension (2 x 10^6/ml) in a horizontal position at 37° C in an atmosphere of 5 % CO_2 in air in an incubator.

C. Results

1. The effect of prolonged culturing of lymphocytes on NK- and K-cell activity

We designed an experiment to study whether the various fractions showing NK- cell activity and containing FcR bearing cells needed the presence and/or functional activity of these FcR.

From table I A it can be seen that prolonged culturing of population A lymphocytes increased the NK-cell activity against the monolayer cell lines tested (table I A, fractions A and A_c, column NK_{lt} cytotoxicity). The NK cell cytotoxicity against K-562 of the cultured fractions (A) from donor 321 was decreased, but there was also a drastic decrease in the percentage EA-RFC (table II A, donor 321, column EA-RFC).

In order to see whether the cytoxicity observed was mediated through the IgG-FcR, the EA-RFC depleted lymphocyte fraction was cultured. As shown earlier, EA-RFC depleted cells, although occasionally remaining cytotoxic against tar-

Table I A

PER CENT CYTOTOXOCITY OF VARIOUS LYMPHOCYTE FRACTIONS AFTER SEPARATION EA-RFC DEPLETED FRACTIONS, EA-RFC RECOVERED FRACTION AND THE EFFECT OF PROLONGED CULTURING ON NK CELL CYTOTOXICITY

	target cells				
		NK_{lt}		NK_{st}	K
lymphocyte donor	NKI-4[1]	Mel-I[1]	colon[1]	K-562[2]	P-815[2]
Donor 313					
A) unfractionated lymphocytes	33*	21*		20*	14*
A_c } unfractionated lymphocytes** cultured for 1 week					
D) EA-RFC depleted fraction	21*	16		4	0
D_c } EA-RFC depleted fraction cultured for 1 week					
E) EA-RFC recovered fraction				47*	8
Donor 314					
A) unfractionated lymphocytes	64*		44*	35*	39*
A_c } unfractionated lymphocytes cultured for 1 week	74*		59*	21*	49*
D) EA-RFC depleted fraction	18		28*	4	5
D_c } EA-RFC depleted fraction cultured for 1 week	48*		61*	9*	48*
E) EA-RFC recovered fraction	99*		20*	53*	10*
Donor 316					
A) unfractionated lymphocytes	23	22*	44*	9*	9*
A_c } unfractionated lymphocytes cultured for 1 week	77*			38*	22*
D) EA-RFC depleted fraction	19			2	0
D_c } EA-RFC depleted fraction cultured for 1 week	56*			23*	5
E) EA-RFC recovered fraction	30*			19*	0

1 lymphocyte target cell ratio: 200 : 1;

2 lymphocyte target cell ratio: 50 : 1; P-815 is sensitized with IgG (see Materials and methods).

* P-value ≤ 0.05

** lymphocytes fraction were EA-RFC depleted after culturing for 1 week immediately before cytotoxicity testing.

Fractions A, A_c, D, D_c and E were isolated and described in Materials and methods.

get cells growing in monolayers (i.e., NKI-4, Mel-I and/or Colon-1), lost their NK-cell activity against K-562. When these cells (fraction D_c) were cultured for 1 week (fraction D_c) they became extremely cytotoxic against K-562 and the cytotoxicity against the monolayer cultures was increased as well: against NKI-4 (donors 314, 316 and 320), against Colon (donors 314 and 320) and against K-562 (donors 316 and 321) as can be seen in tables 1 A and I B, fractions D and D_c, column NK cytotoxicity. The K-cell activity against IgG-sensitized P-815, however, was very low, and so was the number of EA-RFC. Since these two assays require functionally intact FcR the latter two observations again support the conclusion that IgG-FcR, although they may be present on a proportion of the NK-cells, are not involved in the NK-cell cytotoxic mechanism.

Lymphocytes of donor 314 (table I A), however, showed the opposite effect and lymphocytes of donor 320 showed an increased cytotoxic activity as monitored in both the NK- and K-cell assays.

Whether the NK-cell activity of these EA-RFC depleted and cultured cells (table II B, fractions D_c), which showed no NK cytotoxicity before culturing (table II B, fractions D) represent the induction of new subpopulation of NK-cells, which so far remained undetected has to be elucidated. The per cent EA-RFC was always increased slightly after culturing of the EA-RFC depleted cell population. These results do not clearly demonstrate that the IgG-FcR is not involved in the NK-cell lytic mechanism, although it is clear that the increase in NK-cell activity upon prolonged culturing of the lymphocytes does not necessarily coincide with an increase in antibody dependent K-cell killing and vice versa. Moreover, the effect of culturing of lymphocytes on NK- and K-cell killing seems donor dependent.

TABLE I B

PER CENT CYTOTOXOCITY OF VARIOUS LYMPHOCYTE FRACTIONS AFTER SEPARATION EA-RFC DEPLETED FRACTIONS, EA-RFC RECOVERED FRACTION AND THE EFFECT OF PROLONGED CULTURING ON NK CELL CYTOTOXICITY

	target cells				
	NK_{lt}			NK_{st}	K
lymphocyte donor	NKI-4[1]	Mel-I[1]	colon[1]	K-562[2]	P-815[2]
donor 320					
A) unfractionated lymphocytes	15		48*	14*	23*
A_c) unfractionated lymphocytes** cultured for 1 week					
D) EA-RFC depleted fraction	- 3		4	4	0
D_c) EA-RFC depleted fraction cultured for 1 week	45*		58*	18*	20*
E) EA-RFC recovered fraction	30*		37*	12*	23*
donor 321					
A) unfractionated lymphocytes	39*	83*		59*	73*
A_c) unfractionated lymphocytes cultured for 1 week				18*	0
A_c) unfractionated lymphocytes cultured for 1 week, EA-RFC depleted				17*	0
D) EA-RFC depleted fraction cultured for 1 week				22*	0
D_c) EA-RFC depleted fraction cultured for 1 week, EA-RFC depleted				16*	3
E) EA-RFC recovered fraction	63*	91*		31*	57*

1: lymphocyte target cell ratio: 200 : 1;

2: lymphocyte target cell ratio: 50 : 1; P-815 is sensitized with IgG (see Materials and methods).

* P-value $\leqslant 0.05$

** lymphocytes fraction were EA-RFC depleted after culturing for 1 week, immediately before cytotoxicity testing.

Fractions A, A_c, D, D_c and E were isolated as described in Materials and methods.

Table II A

COMPOSITION OF THE MONONUCLEATED CELL POPULATIONS OF THE DIFFERENT FRACTIONS OF THE ISOLATED FRACTIONS

lymphocyte donor	% E-RFC	immunofluorescence				% EA-RFC
		% T^+	% Ig^+	% T^+/Ig^+	% T^-/Ig^-	
Donor 313						
A) unfractionated lymphocytes	71	73	8	1	18	12
A_c) unfractionated lymphocytes cultured for 1 week						
D) EA-RFC depleted fraction	76	89	2	4	5	1
D_c) EA-RFC depleted fraction cultured for 1 week	77	99	0	1	0	
E) EA-RFC recovered fraction	22					4
Donor 314						
A) unfractionated lymphocytes	82					22
A_c) unfractionated lymphocytes cultured for 1 week	80					7
D) EA-RFC depleted fraction	91	94	0	0	6	1
D_c) EA-RFC depleted fraction cultured for 1 week	79					8
E) EA-RFC recovered fraction	35					12
Donor 316						
A) unfractionated lymphocytes	76					18
A_c) unfractionated lymphocytes cultured for 1 week	65					9
D) EA-RFC depleted fraction	93					1
D_c) EA-RFC depleted fraction cultured for 1 week	80					7
E) EA-RFC recovered fraction	54					7

Fractions A, A_c, D, D_c and E were isolated as described in Materials and methods.

Table II B

COMPOSITION OF THE MONONUCLEATED CELL POPULATIONS OF THE DIFFERENT FRACTIONS OF THE ISOLATED FRACTIONS

lymphocyte donor	% E-RFC	immunofluorescence				% EA-RFC
		% T^+	% Ig^+	% T^+/Ig^+	% T^-/Ig^-	
donor 320						
A) unfractionated lymphocytes	80	87	6	1	6	20
A_c) unfractionated lymphocytes cultured for 1 week	83					7
D) EA-RFC depleted fraction	96	96	0	0	4	0
D_c) EA-RFC depleted fraction cultured for 1 week	78					3
E) EA-RFC recovered fraction	80	95	3	0	2	11
Donor 321						
A) unfractionated lymphocytes	88	95	0	1	4	33
A_c) unfractionated lymphocytes cultured for 1 week						5
D) EA-RFC depleted fraction						2
E) EA-RFC recovered fraction	85	83	8	0	9	5

Fractions A, A_c, D, D_c and E were isolated as described in Materials and methods.

2. The NK- and K-cell activity of EA-RFC recovered lymphocytes: functional involvement of the FcR?

We already have shown that EA-RFC when incubated on EA-monolayers, remain attached to the IgG coated E_{sh} ghost monolayer. These cells, overlayered with medium, were incubated for 18 h at 37° C. After incubation the cells that had spontaneously detached from the monolayers were recovered (total yield 21 %, table I, chapter V) and tested for their NK- and K-cell activity against K-562, NKI-4, Colon-1 and against IgG-sensitized P-815 (tables I A and I B, fractions E). Cell surface marker monitoring revealed that, although theoretically 100 % of these cells are expected to bear FcR, only a very small percentage (4 - 12 %) of these "FcR bearing" cells still bear IgG-Fc receptors capable of binding to IgG sensitized E_{sh} and fresh EA-monolayers (table I B and II B, fractions E, column % EA-RFC). If one compares the NK-and K-cell activity (ADCC) against K-562 and IgG sensitized P-315 of the EA-RFC recovered fraction to that of the unfractionated cells, one can see that the lymphocytes of 3 out of 5 donors (donor 313, 314, 316, tables I A and I B, fractions A and E, column NK_{lt} and K) showed an increased NK- but a decreased K-cell activity.

For 2 out of 5 donors the level of NK- and K-cell activity remained the same as before EA-RFC recovery (donors 320 and 321, tables I A and I B, fractions A and E, columns NK_{st} and K). The NK-cells of all donors showed an increased lytic activity against the monolayer target cells (tables I A and I B, column NK_{lt}). When the EA-RFC recovered fractions were depleted for residual EA-RFC directly before cytotoxicity testing, the results became more clear. As can be seen in fig. 1, the levels of cytotoxicity against K-562 and P-815 before fractionation were equal and the influence of the effector target cell ratio was monitored.

When the EA-RFC recovered fraction was depleted for residual EA-RFC directly before the cytotoxicity testing, one can see that the level of NK-cell activity remained unchanged or

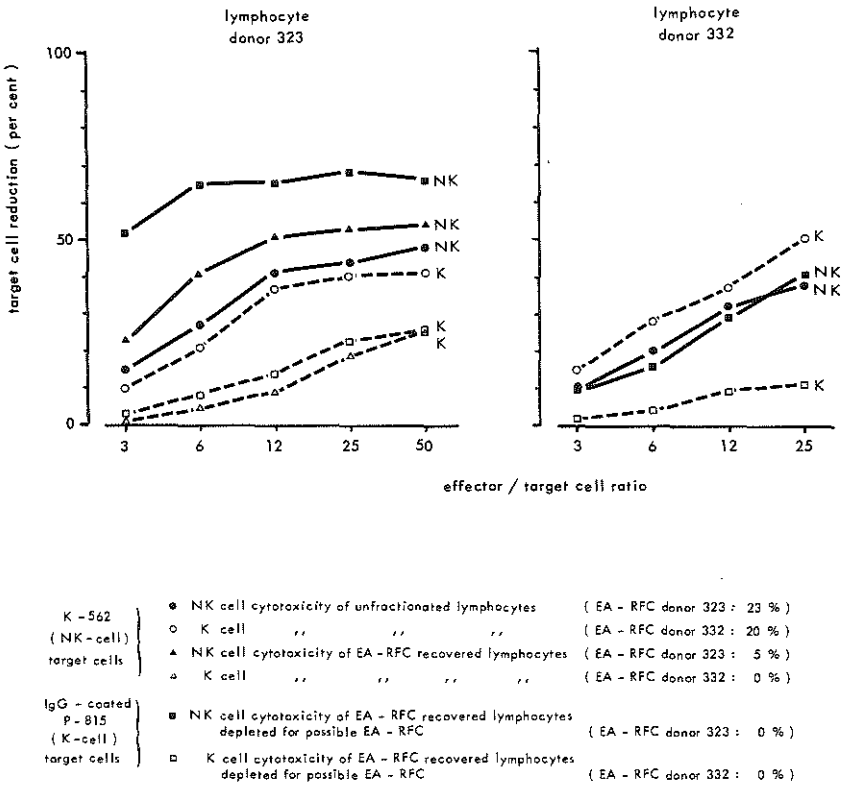


Figure 1. Separation of NK- and K-cell activity by means of EA-RFC recovery followed by EA-RFC depletion.

was even somewhat higher as compared to the unfractionated lymphocytes, whereas the level of K-cell activity was decreased. Furthermore, at an effector target cell ratio of 3 : 1 the NK-cell per cent cytotoxicity against K-562 is still significant whereas the K-cell activity is completely abolished. Moreover, the decrease in K-cell activity coincided with a decrease in the per cent EA-RFC.

3. The influence of soluble complexes on the NK- and K-cell activities

Complexes were expected to reduce the lytic activity of K-cells by "plugging" of the IgG-FcR on the surface of the effector lymphocytes. That this is indeed the case can be seen in fig. 2. The maximally per cent reduction of K-cell cytotoxicity was 100 % for lymphocytes of two of the three donors and 60 % for 1 donor, depending on the effector/target cell ratio. The reduction in per cent NK-cell cytotoxicity, however, is much less, ranging from 40 to 65 per cent, suggesting that at least a subset of the NK_{st}-cells do not need the functional involvement of the IgG-FcR for NK killing of K-562 target cells.

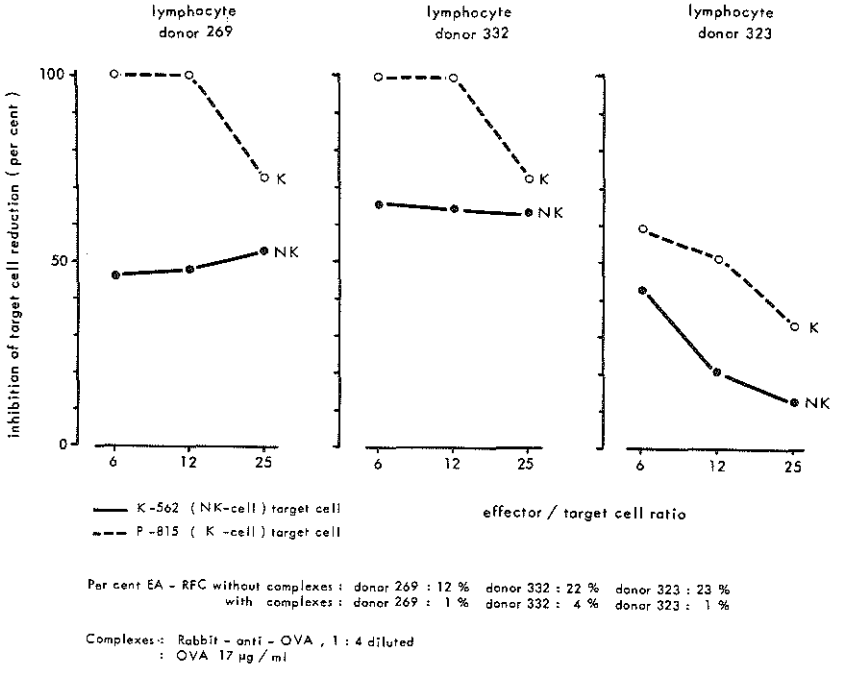


Figure 2. Per cent reduction by immune-complexes of the cytotoxic capacity of NK- and K-cells.

This stronger inhibition of K-cell cytotoxicity is illustrated by a complete inhibition of EA-RFC formation (fig. 2). Figures 3 and 4 show the data obtained from an experiment combining the monitoring of the NK- and K-cell activity of EA-RFC recovered, EA-RFC depleted fraction (described earlier) with inhibition of the NK- and K-cell cytotoxicity by immune complexes. Since the total level of NK-cell cytotoxicity of unfractionated lymphocytes is reduced by the immune complexes, this experiment allows the interesting conclusion that the cytotoxic capacity of a proportion of the NK_{st}-cells can be inhibited by complexes. These NK_{st}-cells thus probably bear FcR on their surface.

The K-cell activity, however, is reduced even stronger. Again, from figs. 3 and 4 it can be seen that addition of immune complexes completely inhibit EA-RFC formation and that no EA-RFC are present in the EA-RFC recovered fraction after IgG-E_{sh} short monolayer incubation. When the influence of the addition of immune complexes on the NK- and K-cell activity of the EA-RFC recovered cell population, after depletion of residual EA-RFC, is monitored one can see that the NK-cell activity is only slightly reduced. This observation allows the conclusion that two subsets of NK_{st}-cells exist: 1. NK_{st}-cells bearing IgG-FcR on the cell surface since this NK-activity can be blocked by immune complexes and hence may require the functional involvement of FcR for its lytic activity; and 2. NK_{st}-cells of which the lytic activity cannot be blocked by immune complexes. The latter cell population expressed IgG-FcR on its surface before fractionation. Thus this IgG-FcR seems not to be functionally involved in the lytic NK process.

An alternative explanation is that the inhibition of NK-cell cytotoxicity by immune complexes was caused by steric hindrence rather than by blocking of a functionally required FcR.

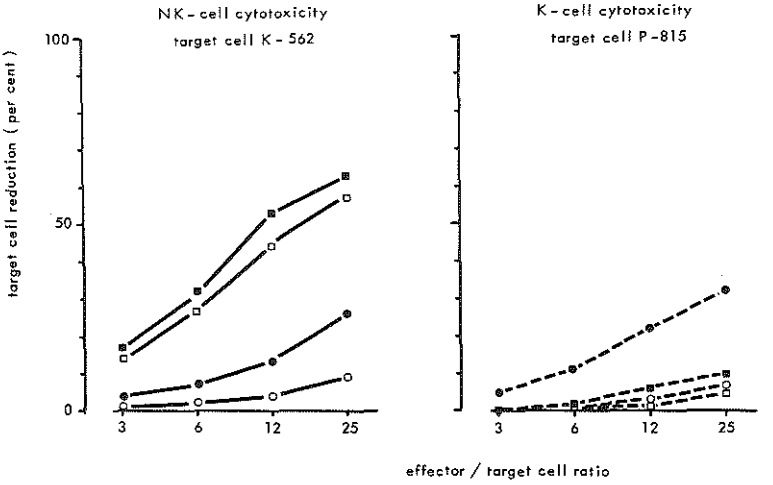
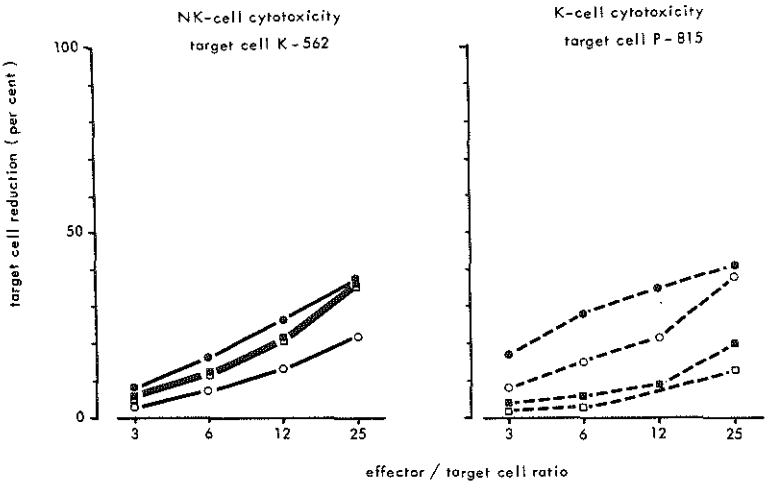


Figure 3. The influence of immune complexes on the NK- and K-cell activities of unfractionated and EA-RFC recovered lymphocytes separation of NK- and K-cell activities



● unfractionated lymphocytes (EA - RFC : 18 %)

○ unfractionated lymphocytes + rabbit - anti - OVA - OVA complexes (EA - RFC : 4 %)

■ EA - RFC recovered lymphocytes (EA - RFC : 0 %)

□ EA - RFC recovered lymphocytes + rabbit - anti - OVA - OVA complexes (EA - RFC : 0 %)

Figure 4. The influence of immune complexes on the NK- and K-cell activities of unfractionated and EA-RFC recovered lymphocytes separation of NK- and K-cell activities

D. Conclusions

As stated in the previous chapter NK-cell killing may require FcR and hence display an antibody dependency for its lytic activity. The antibody may already be present on the cell surface of the purified lymphocytes (*in vivo* coating of lymphocytes by antibodies) or be produced during the 4 h period required for cytotoxicity testing.

Jondal and Pross (1975) showed that the NK-cells separated with the complement receptor bearing cells and not with E-RFC and stated that these effector cells were non-T-cells. Earlier work of these researchers suggested these non-T-, complement receptor bearing cells to bear FcR on their surface (Jondal, 1974). When, however, complement receptor bearing cells were isolated with pure IgM antibodies the NK activity did not separate with the complement bearing cells. Use of 7S IgG, however, yielded similar data as reported by Jondal (West et al., 1977). Most likely the separation of NK-cell activity was mediated through the IgG-FcR. Other investigators confirmed the presence of IgG-FcR on the surface of NK-cells (Peter et al., 1975; West et al., 1977; Kay et al., in the press). Both, NK cells as well as K-cells are reported to bear FcR. The data presented in the previous chapter suggest that another, FcR negative, cell population shows NK-cell activity, but exclusively against monolayer tumour target cells.

It was shown in this chapter that prolonged culturing of lymphocytes, without IgG-FcR, induces IgG-FcR formation or expression and induces NK-cell activity. These observations are in accordance with the data of Ortaldo et al. (submitted for publication). These investigators also showed that this NK-activity is only induced when the culture medium is supplemented with foetal calf serum. Human serum has no such an effect. The MCT, using monolayer cells as target cells, requires 48 h. Thus one may argue that NK-cells are generated during the cytotoxicity assay. Lymphocytes, cultured for 48 h

and subsequently tested for NK-cell activity against monolayer target cells in a 4 h ^{51}Chromium-release assay of monolayer target cells have, however, no lytic effect (unpublished data). Thus, the cytotoxicity observed seems indeed to be effected by a newly detected subset of IgG-FcR negative lymphocytes. Stejskal et al. (1973) reported on induced cytotoxicity after culturing of lymphocytes in foetal bovine serum and others confirmed their observation (Zielske and Golub, 1976; Levin et al., 1976; Bolhuis et al., submitted for publication; Ortaldo et al., submitted for publication; this chapter). The susceptibility of K-562 and P-815 cells to lysis as a result of the induced cytotoxicity after culturing in medium supplemented with foetal calf serum make it probable that their observations represent the induction of NK-activity.

One of the main efforts in the field of tumour immunology has been the direct cytotoxicity testing of lymphoid cells of cancer patients. The aim was to demonstrate that the isolated cells were educated *in vivo* to mediate tumour specific cytotoxic reactions (chapter I).

That this expectation was not satisfied is discussed earlier (chapters I and II). Recently, much attention is given to the indirect cytotoxicity testing. This involves the culturing of lymphocytes with (allogeneic) tumour cells with subsequent monotoring of cell mediated cytolysis of the tumour cells. If one chooses such an approach it has to be realized that foetal calf serum in the culture medium rather than the tumour cells would induce the cytotoxicity observed later during the cell mediated cytotoxicity phase of the experiment and thus foetal calf serum is not recommended for this type of experiment.

The issue remains whether the NK-cells with IgG-FcR on their surface can be functionally equated with the IgG-FcR bearing K-cells. This would implicate the functional involvement of the IgG-FcR in the NK lytic process. The results obtained from the cytotoxicity testing of fractionated lympho-

cytes cultured for 1 week already indicated that the NK-cell activity did usually not separate with the K-cell activity. The EA-RFC recovered lymphocyte population of 3 out of 5 donors showed increased NK-cell activity without a concomitant increase in K-cell activity. No trypsin was used for the recovery of the EA-RFC cells since this might lead to inactivation of NK-cells (Jondal and Pross, 1975; Faulk and Hijmans, 1972). Moreover, these cells beared IgG-FcR on their surface before fractionation, since they were repeated on the basis of that quality. After 18 h incubating, however, low numbers of FcR bearing cells were detected, probably because the cells had shed off their FcR. Again this observation suggests that NK- and K-cells are distinct cell types or when there is an overlap between these cell types (K-cells and NK_{lt}- or NK_{st}-cells, FcR positive) the NK- and K-cell lytic capacity are distinct functions of the individual lymphocyte.

The immune complex inhibition experiments of NK- and K-cell cytotoxicity and the combination of NK or K cytotoxic tests of EA-RFC recovered cells with immune complex inhibition provides experimental basis for the hypothesis presented here i.e., that several types of NK-cells exist. Whether the lytic mechanism of a subset of these NK-cells is antibody dependent as suggested by others (Saal et al., 1977; Akira and Takasugi, 1977; Blair and Lane, 1975) needs further experimentation.

GENERAL DISCUSSION

The importance of the immune system as a defense mechanism against the proliferation of cancer cells has been considered during the past 20 years ranging from having no role at all to it is the major defense mechanism. Others now say that it might be a mechanism of questionable importance. Many clinicians and researchers took the newly formulated theory of immunosurveillance on the status of a dogma and numerous examples could be found in the literature where the unique antigenicity of tumour cells was demonstrated in experimental animal systems. The enthusiasm about these reports, however, obscured the boundaries between expectations and facts once the search for such tumour associated antigens was initiated in human tumour systems. Transformation from normal to neoplastic cells involves a number of changes such as alterations of the composition and arrangement of cell surface components and any such variations were thought to lead to the appearance of new antigenic structures at the surface of these cells.

We have been able to demonstrate that there is no clear evidence at present for the existence of tumour associated antigens in the sense that these are specific for tumours of a certain histologic origin. The inability to demonstrate the presence of tumour associated antigens was due to heterogeneity of the material (chapter II), the fact that the interpretation of results was compromised by the selection of lymphocyte control and/or target cell control, the changes in susceptibility to lysis of the target cells as a result of subculturing and the lack of reproducibility of the assay. Furthermore, the NK-cell activity may obscure the detection of tumour-specific reactions. Hence, our findings do not implicate that there are no such antigens or that there is

no role for the immune response in modulating malignant disease in a profitable sense for the tumour bearing individual.

Many if not all naturally occurring immunogenic tumours of animals may turn out to have a viral etiology resulting in the neo-antigenicity of the malignant cells. There are indications that also viruses play a role in a variety of spontaneous cancers in man (Nooter et al., 1975). However, the tumours of laboratory animals naturally infected with oncogenic viruses express antigens of which the antigenicity is several orders of magnitude higher than those found on human tumours with a suspected viral etiology. Hence human tumours may have only a marginal expression of these antigens which are below the threshold of active immunogenicity.

The partial development of specific immunological tolerance to viral associated tumour antigens in a long infected host must be considered as another possibility.

Little is known about the antigenicity of spontaneous tumours in wild mice and rats which probably have a viral etiology. It would be of great importance to study the antigenicity of these tumours. In the introduction the assumption has already been discussed that frequent natural exposure of animals to viruses has selected the host for high immune responsiveness and therefore one could envisage in the human situation that there may be an imperfect genetic selection for resistance against tumours developing early at life due to the fact that the infrequent occurrence does not result in such a selective pressure. For the majority of spontaneous tumours in adult man it is known that they appear late in or after the reproductive period. Therefore, a selective evolutionary pressure for the development of high responsive tumour specific immune recognition systems seem unlikely.

Clear evidence was given in chapters V and VI for the presence in human peripheral blood of natural killer cells against monolayer cultures. Preliminary experiments suggest that this natural activity exhibits immunologic selectivity. It was further shown that the natural killer cytotoxicity is

exerted by various cell populations, i.e., T-cells with or without IgG-Fc receptors and furthermore that T-cells without IgG-Fc receptors exhibited their cytotoxic effect against monolayer target cells but not against myelogenous leukaemia cells growing in suspension. Although this natural killer cell phenomenon has obscured the detection of tumour specific immune responses in human systems we have to recognize the possible important *in vivo* surveillance function of these cells.

The main question is whether it will be possible to modulate the immune response in such a way that it can effect spontaneously arising tumours in man, tumours which only slightly differ from analogous normal cells in antigenic surface structure and which are only weakly immunogenic in the tumour bearing individual. This question may prudently answered positively and although highly speculative, without firm experimental evidence, there are several approaches to tumour prophylaxis and immunotherapy which are open for experimentation.

1. Nonspecific immunotherapy

Immunotherapy of human cancer has become an important modality in clinical oncology. A wide variety of microbial substances has been investigated for their capacity to affect immunological responsiveness at various stages. They can elevate the immune response against otherwise very weak antigens by combining with the antigen or altering its antigenicity. Weiss (1972; 1976) has studied in detail the behaviour of such an adjuvant or better immune modulator, the methanol extraction residu fraction of tubercle bacilli. This study indicated the multifaceted applicability of such reagents to the prevention and treatment of malignant disease. It was shown that this immunomodulator enables an organism to respond to otherwise non-immunogenic substances or even prevent or break a state of specific immunological tolerance. If one takes into account the lack of strong immunogenic antigens on the

surface of tumour cells and the possibility of tolerance for tumour associated antigens it is obvious that these immune modulators can be of crucial importance in developing resistance of the tumour host against progressive neoplasia. The importance for chemical and physical characterization of immunomodulators and the association of the immunological activity with structure and physical state cannot be emphasized enough.

2. Structural modifications of weak antigens

There are a number of ways in which immunogenic modifications of antigen can be brought about, for instance, by chemical and enzymatic treatment or by cellfusion techniques. Such modifications have been shown to provoke new immunogenic qualities or immunogenicity and to break states of specific immunological unresponsiveness (Prager, 1973). It will be of great interest to investigate whether animals immunized with tumour cells with new or altered antigenic structures induced either by chemical compounds, viruses or cell fusion can then recognize the non-immunogenic structures present on the surface of the unmodified tumour cells. Previous experiments have shown that *in vitro* education of human lymphocytes on monolayers of tumour cells results in the generation of nonspecific killer cells. This may be explained by the induction of natural killer cells after culturing the cells as described in chapter VI. If tumour cells with an altered antigenic structure as described above may become immunogenic one could envisage the possibility of *in vitro* education of autogenous and allogeneic effector cells for passive adoptive immunization.

SUMMARY

In chapter I the pro's and con's regarding immunosurveillance were discussed and it is clear that no conclusive evidence can be given to either approve or disapprove the validity of the concept.

Each tumour-host system will have to be analysed on its own merit, and the importance of the immune system as a defense mechanism against cancer will depend on the etiology of the various forms of malignancies.

The core question of tumour-immunologists today is whether the hope can be entertained for a successful immunologic approach in the control or cure of cancer in man, especially since these tumour cells seem to differ only slightly in antigenic surface structure from analogous normal counter parts.

The author contends that this question can prudently answered in the affirmative when in spite of all the justified hurry, serious assessment will be allowed for the molecular characterization of tumour antigens and a profound investigation of the host capacity to respond to these antigens, so that more than faith alone will be the basis of future attempts at immunotherapy.

In chapter II the use of the microcytotoxicity test is described to determine the cytotoxic effect of purified peripheral blood lymphocytes from patients with carcinoma of the urinary bladder, tumour control patients and healthy donors against cultured bladder cancer, melanoma and "normal" bladder epithelial cells. Disease specific cytotoxicity is defined as statistically significant and selective destruction of disease-related tumour target cells. Within the different donor groups, an enormous variation in non-specific cytotoxic effects of the lymphocytes against the various cell

lines was seen. It appeared that the selection of the baseline control and of type of target cell control influences the level of cytotoxicity and interpretation regarding the specificity of the reactions observed. In order to determine whether a tumour-specific reaction was superimposed on the non-specific natural killer cell activity, the overall cytotoxic effects from the three lymphocyte donor groups were compared statistically. The analysis of results revealed that effector cells from bladder cancer patients and tumour control patients, when tested after therapy showed the same pattern of reactivity of healthy donors. The cytotoxicity of lymphocytes from bladder cancer patients, when tested before the initiation of therapy, was stronger in comparison with the other two groups of donors, but this stronger cytotoxic reactivity against tumour cells was not tumour-specific.

In chapter III the statistical analysis of the reproducibility of the microcytotoxicity test is described. It was concluded that the experiment-to-experiment variation, using the same cryopreserved effector and target cells repeatedly, is large and hence does not allow longitudinal monitoring of the cytotoxic capacity of patients lymphocytes in independent experiments. Moreover, it was concluded that cryopreservation in general decreases the lytic capacity of lymphocytes and increases the lysibility of target cells.

In chapter IV the identification of a new subpopulation of human T-cells, carrying a receptor for the Fc-portion of IgM type antibodies is described. It was shown that either free IgM antibodies or IgM-antibody-antigens complexes can be bound to the receptor on the surface of the lymphocytes.

The specificity of the binding was demonstrated by the fact that either IgM or Fc-fragments of IgM antibodies could block the binding capacity of the receptor, but not IgG.

Moreover, the pentameric structure of IgM needs to be preserved for binding, since cleavage products do not bind to the receptor.

In chapter V the nature of the natural killer cells was investigated. Analysis of cell surface markers resulted in the following conclusions: 1. NK-cell belong to the T-cell subset of lymphocytes; 2. B-cells are not involved in the NK cytotoxic mechanisms as studied in the assays described; 3. monocytes are not involved in the cytolytic process of NK-cells.

In chapter VI the analysis of the functional involvement of the IgG-Fc receptor was explored. When IgG-FcR bearing cells, absorbed onto plastic surfaces covered with IgG coated E_{sh} membranes, were recovered from these monolayers by 18 h incubation at 37° C, almost no FcR could be detected by means of rosetting techniques. Furthermore, these cells showed a significantly reduced K-cell activity (ADCC). On the contrary, the level of NK activity remained equal or was increased. These results indicate that although both NK-cells and K-cells bear FcR, FcR are not functionally active in NK-cell killing as opposed to the antibody-dependent-cellular cytolysis, illustrating that ADCC and NK-cell killing most likely involve distinct mechanisms. The conclusion was confirmed by the results of another experiment, showing that immune complexes inhibited K-cell activity to a significantly larger extend than NK-cell activity. The NK-cell activity of EA-RFC recovered cells were not inhibited by immune complexes, whereas the K-cell activity was decreased.

SAMENVATTING

In hoofdstuk I zijn de argumenten voor en tegen de immuunsurveillance theorie besproken en het is duidelijk geworden dat er tot op heden geen sluitend bewijs geleverd is, hetwelk de waarde van het concept kan bevestigen of ontkennen.

Ieder tumor-gastheer-systeem zal afzonderlijk op zijn merites als experimenteel model voor kankeronderzoek getoetst moeten worden en het belang van het immuunsysteem als een afweermechanisme tegen kanker zal mede afhangen van de etiologie van de verschillende vormen van maligniteiten.

Voor de tumor-immunoloog is momenteel de belangrijkste vraag of er wel een basis is voor een gerechtvaardigde hoop voor een succesvolle immunologische benadering teneinde controle over of zelfs genezing van het kankerproces bij mensen te bewerkstelligen, juist omdat de antigene structuur van deze tumorcellen slechts in geringe mate verschilt met die van de analoge normale cellen.

De auteur is van mening dat deze vraag voorzichtig bevestigend beantwoord kan worden wanneer, ondanks alle gerechtvaardigde haast, een serieus onderzoek naar de moleculaire karakterisering van tumor-antigenen en een grondig onderzoek van de immuuncapaciteit van de patiënt om tegen die antigenen een respons te geven mogelijk zal worden gemaakt. Dan zullen veeleer resultaten dan geloof de basis vormen voor toekomstige pogingen tot immuuntherapie.

In hoofdstuk II is de toepassing beschreven van de microcytotoxiciteitstest voor het bepalen van de lytische capaciteit van gezuiverde lymfocyten uit het perifere bloed van patiënten met blaaskanker, patiënten met andere vormen van kanker en gezonde donoren op gekweekte blaaskanker-, melanoom- en "normale" blaasepitheel-cellen. Tumorspecifieke cy-

totoxiciteit is gedefinieerd als een statistisch signifikante en selectieve lyseren van de betreffende tumorcellen. Binnen de verschillende groepen donoren werd een enorme spreiding in de non-specifieke cytotoxische effecten van de lymfocyten tegen de verschillende cellijnen waargenomen. Het bleek dat de selectie van de achtergrondcontrole en van het type tumorcelcontrole zowel het niveau van de cytoxiciteit als de interpretatie betreffende de specificiteit van de immuunrespons beinvloedt.

Teneinde te bepalen of een tumorspecifieke reactie gesuperponeerd was op de non-specifieke "natuurlijke killer" celaktiviteit werden de totale cytotoxische effecten van de drie groepen donoren statistisch met elkaar vergeleken. Analyse van de resultaten onthulde dat de effector cellen van blaaskanker patiënten en tumorcontrole patiënten eenzelfde patroon van reactiviteit hadden, wanneer deze getest werden na behandeling. Het cytotoxische effect van lymfocyten van blaaskankerpatiënten, indien getest vóór de aanvang van therapie, was groter in vergelijking met de twee andere groepen van donoren, maar deze grotere cytotoxische reactiviteit tegen tumorcellen was niet tumorspecifiek.

In hoofdstuk III is de statistische analyse van de reproduceerbaarheid van de microcytotoxiciteitstest beschreven. De experiment-tot-experiment variatie bleek groot, ondanks het feit dat dezelfde cryogepreserveerde effector en tumor cellen werden gebruikt. Een regelmatig meten van de cytotoxiciteitcapaciteit, in onafhankelijk experimenten, van lymfocyten van patiënten is dus niet mogelijk. Bovendien werd geon ludeerd dat cryopreservering in het algemeen de lytische capaciteit van lymfocyten doet verminderen en de lyseerbaarheid van tumorcellen doet toenemen.

In hoofdstuk IV is de identificatie van een nieuwe subpopulatie van humane T-cellen met een membraan-gebonden receptor voor het Fc-gedeelte van IgM antilichamen beschreven.

Er werd aangetoond dat zowel vrije IgM antilichamen, als IgM-antilichaam-antigeen complexen kunnen binden aan de receptor op de lymfocytmembraan.

De specificiteit van deze binding werd geillustreerd door het feit dat zowel IgM als Fc fragmenten van IgM, maar niet IgG, binding van de IgM-antigeen complexen aan de Fc receptor konden remmen. Bovendien bleek het intakt zijn van de pentamere struktuur van het IgM een voorwaarde voor binding aan de receptor.

In hoofdstuk V werd de aard van de "natuurlijke killer" cellen onderzocht. Analyse van membraanstrukturen gaven aanleiding tot de volgende conclusies: 1. NK-cellen behoren tot de T-cel subpopulatie van lymfocyten; 2. B-cellen zijn niet betrokken bij het NK-cytotoxicische mechanisme, bestudeerd in de beschreven experimenten; 3. monocyten zijn niet betrokken bij het cytolytische proces van NK-cellen; 4. Een deel van de NK cellen heeft een IgG-Fc receptor op de membraan.

In hoofdstuk VI is de analyse van het functioneel betrokken zijn van de IgG-Fc receptor bestudeerd. Wanneer cellen met IgG-Fc receptor op de membraan, na adsorptie aan IgG bedekte plastic petrischalen, werden teruggewonnen door deze schalen met cellen 18 uur te incuberen bij 37^{o} C kon de vrijwel geen Fc receptoren meer gedecteerd worden op de membraan van deze cellen. Bovendien bleken deze cellen een signifikant lagere K-cel aktiviteit te vertonen. Het niveau van NK-cel aktiviteit daarentegen bleef gelijk of nam toe. Deze resultaten geven aan dat alhoewel NK- zowel als K-cellen een Fc receptor hebben, deze Fc receptoren niet functioneel betrokken zijn bij het NK-cel lytische proces, dit in tegenstelling tot de betrokkenheid van de Fc receptor bij het antilichaamafhankelijke cellulaire cytolytische proces (K-celaktiviteit).

Dit illustreert dat antilichaamafhankelijke cellulaire cytolytische proces en het NK-cel cytotoxische proces ver-

schillende mechanismen vertegenwoordigen. Deze conclusie werd bevestigd door de resultaten van een ander type experiment hetwelk aantoonde dat immuuncomplexen de K-cel reactiviteit signifikant sterker remmen dan de NK-cel aktiviteit. De NK-cel aktiviteit van EA-Fc receptor-geisoleerde cellen werd niet geremd door immuuncomplexen, terwijl de K-cel aktiviteit verminderde.

ACKNOWLEDGEMENTS

The author wishes to convey thanks to:

- Miss H.R.E. Schuit, Mrs. J.M. Nooyen and Mr. C.P.M. Rondeltap for superb technical assistance and collaboration.
- Dr. J. Radl, Miss P. van den Berg, Dr. J. Molenaar and Dr. F. Gmelig-Meyling for providing conjugates and antisera.
- Dr. J.J. Haaijman for his support, discussions and statistical advice for data presented in chapter III.
- Dr. W. Hop for statistical advice for data presented in chapter III.
- Dr. A. Polak-Vogelenzang for checking the cultures for mycoplasma.
- Dr. W. Zeylemaker for discussions regarding the K-cell system and for supplying the mouse mastocytoma cell line.
- Mr. P.E.W. Kuijpers for keeping the material mobile.
- Prof. Dr. B.P. van der Werff-Messing for her interest in tumour immunological research.
- Prof. Dr. D.W. van Bekkum, Prof. Dr. O. Vos and Dr. W. Hijmans for critically reading the manuscript.
- Prof. Dr. D.W. van Bekkum for his hospitality and providing the laboratory and facilities in the Radiobiological Institute TNO.
- Drs. Ph. Rümke and J. de Vries for discussions.
- Dr. W.I. Brouwer for management support.
- My colleagues in the Rotterdam Radio-Therapy Institute for their co-operation in the patient related studies.
- Technical and scientific members of the REPGO-TNO Institutes, who made this thesis possible.

- Mr. J.Ph. de Kler for the preparation of the figures.
- Mrs. Mea van der Sman for telling me in the morning what I wrote the night before and typing the manuscript with verve.

The work presented in this thesis was supported by a grant of the "Koningin Wilhelmina Fonds" of the Dutch National Cancer League for the investigation entitled: Immune resistance against tumours.

LIST OF ABBREVIATIONS

ADCC	antibody-dependent-cellular cytolysis
ALS	anti-lymphocyte serum
BT	bladder cancer
C	Celsius
Cr	Chromium
CTX	cytotoxicity
DMSO	dimethylsulfoxide
DTE	dithioerythritol
E_{sh}	sheep red blood cells
EA	erythrocyte-antibody complex
E-RFC	rosette forming cells (lymphocytes) with E_{sh}
EA-RFC	rosette forming cells with EA
Fc	Fc-portion of immunoglobulin
FITC	fluoresceine isothiocyanate
Ig	immunoglobulin
HD	healthy donor
lt	long term
Ly	lymphocytes
Ly-IgM	lymphocytes complexed with IgM
min	minutes
MCT	microcytotoxicity test
MSV	mouse sarcoma virus
NK	natural killer
nu/nu	nude mice
RFC	rosette forming cells
st	short term
TA(T)A	tumour-associated (transplantation) antigens
TRITC	tetramethyl rhodamine isothiocyanate

Cell lines

T24	bladder cancer cell line
Colon-1	colon cancer cell line
MEL	melanoma cell line
HCV	bladder epithelium cell line

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