

**NATURAL ANTIBODY ANTI-TUMOR RESISTANCE ACTING THROUGH
CD45**

By

ZIYUAN ZHANG

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**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University
of Manitoba in partial fulfillment of the requirements of the degree**

of

MASTER OF SCIENCE

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To my wife and my daughters

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LIST OF ABBREVIATIONS

AA: amino acids

ADCC: antibody dependent cell-mediated cytotoxicity.

ALL: acute lymphoblastic leukaemia.

AML: acute myeloid leukemia.

B-CLL: chronic lymphoblastic leukemia.

CLL: chronic lymphocytic leukemia.

CMC: cell-mediated cytotoxicity.

CSF: cerebrospinal fluid.

CTL: cytotoxic T lymphocytes.

DMEM: Dulbecco's Modified Eagle Medium.

EAE: experimental allergic encephalomyelitis.

ECL: enhanced chemiluminescence.

EGTA: ethylene glycol bis(aminoethyl ether)N N,N'N'-tetraacetic acid;

EDTA: ethylenediamine-tetraacetic acid.

ET: Ehrlich tumor.

DMSO: dimethylsulfoxide.

FACS: fluorescence-activated cell-sorting.

FBS: fetal bovine serum.

FM: Fischer's medium.

GVHD: graft-versus-host disease.

HBSS: Hanks' balance salt solution.

HIV: human immunodeficiency virus.

HMCL: human melanoma cell line.

HRP: horseradish peroxidase.

ICs: immune complexes.

i.p.: intraperitoneal.

i.v.: intravenous.

IVIg: intravenous immunoglobulin.

LCA: leukocyte common antigen.

LPS: lipopolysaccharide.

MCF: mean channel fluorescence.

MAB: monoclonal antibody.

MDS: myelodysplastic syndrom.

MLR: mixed lymphocyte reaction.

MM: multiple myeloma.

MS: multiple sclerosis.

NAb: natural antibody.

NB: neuroblastoma.

NHL: non-Hodgkin's lymphoma.

NHS-biotin: N-hydroxysuccinimido-biotin.

NK: natural killer

NR: natural resistance.

PBMC: peripheral blood mononuclear cells.

PBS: phosphate-buffer saline.

PE: phycoerthrin.

PMSF: phenylmethylsulfonyl fluoride.

PTKase: protein tyrosine kinase.

PTPase: protein tyrosine phosphatase.

RA: rheumatoid arthritis.

RBC: red blood cells.

RT: room temperature.

SAS: saturated ammonium sulfate.

s.c.: subcutaneous.

scid: severe combined immunodeficient disease.

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

SLE: system lupus erythematosus

TAA: tumor-associated antigens.

T-ALL: T-acute lymphoblastic leukemia.

TAP: transporters associated with antigen processing.

TBST: Tris buffer solution-Tween 20

TCR: T cell receptor.

t_d : t-dependent.

t_i : t-independent.

TIL: tumor infiltrating lymphocytes.

TPA: 12-0-tetradecanoylphorbol-13-acetate.

TSH: thyroid stimulating hormone.

WT: wild type.

ABSTRACT

There is considerable evidence supporting the idea that polyclonal serum natural antibody (NAb) plays a role in the mediation of natural resistance against tumors. However, the molecular basis of NAb activity remains unknown. Selection of murine T-lymphoma cells L5178Y-F9 for high NAb binding gave rise to the variant LYNAb⁺ which exhibited a reduced tumorigenicity compared to parental cells. Accompanying the increased NAb binding, LYNAb⁺ bound more monoclonal antibody (mAb) 14.8 anti-CD45RA and DNL-1.9 anti-CD45RC, less I3/2 anti-pan CD45 and the binding of MB23G2 anti-CD45RB was eliminated. Neuraminidase treatment to remove sialic acid increased NAb binding and the expression of pan CD45, CD45RA and CD45RC but reduced CD45RB expression, indicating that the epitopes recognized by the former antibodies are altered by sialic acid, while the latter is dependent upon sialic acid. In vivo passage of the LYNAb⁺ as a threshold s.c. inoculation in syngeneic DBA/2 mice yielded more tumorigenic variants, X1SC, X2SC and X3SC, all of which bound less NAb, anti-CD45RA and anti-CD45RC mAb, more anti-pan CD45 mAb but did not bind anti-CD45RB mAb. In agreement with the mAb binding, the immunoprecipitation assay showed that the L5178Y-F9 and an in vivo passaged LYNAb⁺ variant expressed predominantly lower molecular weight CD45 isoforms while the LYNAb⁺ expressed mainly 200 KD isoforms. The consistently parallel alterations in CD45RA and CD45RC expression and NAb binding, corresponding with inverse changes in tumorigenicity exhibited by high NAb binding variants selected in vitro or through tumor progression in vivo, suggests that NAb binding is positively correlated with the expression of asialo high molecular weight isoforms of CD45. Furthermore, CD45⁺ YAC-wild type and CD45

transfected CD45⁻ YAC cells bound more NAb than the CD45⁺ YAC cells, and purified natural IgG inhibited anti-CD45RA mAb binding by 26% indicating that CD45 may bind CD45. In addition, purified CD45RABC and CD45RBRO inhibited natural IgG binding to cells by 36.9% and 34.2%, respectively. Therefore, this study demonstrates that NAb binding and CD45RA and RC expression are inversely associated with tumorigenicity, and NAb from normal mice serum may recognize CD45RA on the cell surface. This study may also suggest that NAb functions in the regulation of T cell activation and anti-tumor resistance through CD45.

1. INTRODUCTION

Although NABs have been studied for several decades (reviewed by Avrameas, 1991; Baumgarth *et al.*, 2000), there are relatively few studies regarding their mechanism(s) of anti-tumor resistance. It has been commonly accepted that NAB acts in a first line of the natural defense system (Ochsenbein *et al.*, 1999), but the real mechanism is still unknown. Most early clinical studies on NAB focused on autoantibodies against self-antigens in autoimmune diseases. Nowadays, more and more studies have found that intravenous immunoglobulin (IVIg) can regulate the immune system (Shimozato *et al.*, 1990; Toungouz *et al.*, 1995; Aktas *et al.*, 2001). NAB in the normal sera from healthy individuals can inhibit the growth of human tumors in animal models. (Ollert *et al.*, 1996; Shoenfeld and Fishman, 1999). The concept that NAB plays a role in anti-tumor resistance has been widely accepted (Bohn, 1999). Researchers are increasingly interested in the investigation of mechanisms of NAB function, such as the NAB binding molecules, especially cell surface molecules (David *et al.*, 1999).

Our lab has been studying anti-tumor NAB for 20 years. More and more publications have confirmed our observations that NAB contributes to the anti-tumor defense mechanisms. Our increasing interest was in finding NAB binding targets. A T lymphoma cell line L5178Y-F9 was selected for high NAB binding by FACS and a cell line LYNAb⁺ was obtained (Tough and Chow, 1988). Accompanied with increased NAB binding on LYNAb⁺, it also showed increased expression of several molecules including CD45RA (Chow *et al.*, 1999). Another experiment by Chow showed that NAB binds to

purified CD45RBRO and CD45RABC by ELISA (unpublished data). These experiments suggested that CD45RA might be one of the targets of NAb binding molecules. Since CD45 is a highly glycosylated transmembrane protein expressed on almost all hematopoietic cells and it may be an important molecule involved in the tumor transformation, further investigation of the relationship of NAb binding and CD45 expression may lead us to understand the mechanisms of NAb in immune surveillance and natural defense against early tumor development. Therefore, this study was to find more evidence that CD45 may be one of the targets on the cell surface of NAb binding cells. We compared the correlated changes of NAb binding and CD45RA expression 1) between the parental L5178Y-F9 and its high NAb binding variant LYNAb⁺, 2) between LYNAb and its *in vivo* selected cells, and 3) between CD45 negative YAC-N1, YAC-WT and CD45 transfected YAC-33 cells. Then, we used NAb to inhibit anti-CD45 mAb binding. Finally we used purified CD45RABC and CD45RBRO and tested their capacity to inhibit NAb binding to LYNAb⁺.

1.1. IMMUNE SURVEILLANCE AND EARLY TUMOR DEVELOPMENT

Cancers are diseases in which a single cell acquires the ability to proliferate abnormally, resulting in an accumulation of progeny (Vogelstein and Kinzler, 2002). Immune surveillance is defined as the role that the immune system plays in the recognition and destruction of cancer cells before they grow into tumors (Abbas *et al.*, 1994). B cells, helper T cells, cytolytic T lymphocytes (CTL) (Cox *et al.*, 1994), natural

killer (NK) (Garrido *et al.*, 1997) cells and NAb are thought to contribute to immune surveillance. In the past several decades, huge amounts of studies have been done on the role of these cells against tumor development and growth. However, we still do not fully understand how tumors escape from the immune surveillance.

1.1.1. How tumors escape immune surveillance.

Multiple factors contribute to the escape of tumor cells from immune surveillance. It has been considered that tumorigenesis represents an imbalance between cell birth and cell death. Oncogenes and tumor suppressor genes directly control cellular proliferation, and the mutation of these genes leads to accelerated tumorigenesis (Vogelstein and Kinzler, 2002). Five different types of genetic alterations have been found in tumor cells: 1) small deletions, insertions and single base-pair substitutions; 2) somatic losses or gains of chromosomes; 3) chromosome translocations; 4) gene amplifications; and 5) introduced exogenous genes, such as from tumor viruses (Vogelstein and Kinzler, 2002).

It is established that tumor recognition and rejection are mediated by MHC class I-restricted CTL (Cox *et al.*, 1994). Tumor cells can present tumor-associated antigens (TAA) in the context of MHC molecules that can be recognized by CTL (Boel *et al.*, 1995). A number of TAA have been identified on human melanoma cell lines and can be recognized by CTL (Cox *et al.*, 1994; Gaugler *et al.* 1994; Boel *et al.*, 1995). However, treatment of human cancer by administration of TAA as potential immunogenic vaccines has achieved very limited success. It has been considered that the defects in MHC class I-

restricted antigen processing in tumor cells contribute to the escape of tumor cells from immune surveillance, leading to the resistance to CTL-mediated recognition (Garrido *et al.*, 1997; Khanna, 1998). These MHC defects include the down-regulation of the multicatalytic proteasome complexes LMP2 and LMP7 and the transporters associated with antigen processing TAP-1 and TAP-2, plus reduced MHC class I expression (Khanna, 1998). It has also been found that CTLs are not tolerized or deleted, because immunization with tumor cells is still able to induce a CTL response (Ochsenbein *et al.*, 1999). Immunologic ignorance may contribute to the escape of immune surveillance due to the long-time growth of tumors in immunoprivileged sites (Ochsenbein *et al.*, 1999).

NK cells also participate in anti-tumor surveillance. In contrast to CTL, reduction in MHC class I expression increases susceptibility to NK cell mediated cytotoxicity (Garrido *et al.*, 1997). Markel *et al* reported that homotypic CD66a interaction between a melanoma cell line and NK cells inhibited the cytotoxic activity of NK cells (Markel *et al.*, 2002). Down-regulation of CD1d expression of tumor cells reduced the immune recognition by NK cells (Fiedler *et al.*, 2002). In addition, some studies have shown that HLA-G, a non-classical MHC molecule, may protect tumors from lytic activity of both NK cells and CTL cells (Riteau *et al.*, 2001). The expression of MUC1, a large mucin molecule also contributes to the immune escape. Expressed by several tumors, MUC1 interferes with normal intercellular adhesion, resulting in the protection of tumor cells from the killing by CTL and NK cells (Zhang *et al.*, 1997). MUC1 also suppresses the proliferative response of T cells (Agrawal *et al.*, 1998). Another process of immune escape is the inhibition of the activation and proliferation of immune cells by stimulating

the production of immunosuppressive cytokines from lymphocytes, such as TGF- β (Chouaib *et al.*, 1997), IL-10 (Maeda and Shiraishi, 1996).

There are few studies on how tumors escape from antibodies. One study showed that the specific IgM was absorbed by the carbohydrate shed from Ehrlich tumor (ET) cells during the tumor progression (Vinuela *et al.*, 1991). The serum from patients with multiple endocrine neoplasia type 1 prolactinoma contains natural autoantibodies which mimicked basic fibroblast growth factor (Zimering and Thakker-Varia, 2002). Basic fibroblast growth factor is a potent tumor angiogenesis factor, not existing in serum of normal individuals (Zimering and Thakker-Varia, 2002)

1.2. NATURAL HUMORAL DEFENSE SYSTEM AGAINST TUMORS

1.2.1 Natural antibody

Natural antibody (NAb) is defined as the antibody in the serum of healthy individuals in the absence of deliberate immunization with target antigens (Lacroix-Desmazes *et al.*, 1998). NAb has been detected in the circulation and peritoneal fluid of almost all vertebrate species (Avrameas, 1991; Baumgarth *et al.*, 2000). Although NAb are often considered to be antibodies derived from heritable or germline genes (Calvanico *et al.*, 1993; Ochsenbein and Zinkernagel, 2000; Ricki, 1994), some antibodies which are induced by exposure to microbes and food antigens and are not encoded by germline genes are also considered to be NAb (Avrameas, 1991). CD5⁺ B1 cells in humans or Ly-

1^+ B cells of mice are the major source of NAb (Bikah *et al.*, 1996), although $CD5^-$ B2 cells also produce NAb (Kasaian *et al.*, 1992). It has been shown that NABs produced by both $CD5^+B1$ cells and $CD5^-B2$ cells exhibit the same primary amino acid sequence in their CDR3 and both cell types equally contribute to the production and polyreactivity of NAb (Kiyoi *et al.*, 1995; Ye *et al.*, 1996). NAb is mainly polyreactive IgM, though polyreactive IgG and IgA also exist in blood circulation (Avrameas, 1991, Ochsenein and Zinkernagel, 2000). Secretory natural IgA (sIgA) has also been detected in saliva and colostrums (Quan *et al.*, 1997). NAb exhibits a wide range of affinity, ranging from $K_d=10^{-5}$ to $K_d=10^{-10}$ M/L (Kasaian *et al.*, 1992; Adib-Conquy, 1993; Diaw *et al.*, 1997).

The polyreactivity of NAb is characterized by its reaction with multiple structurally unrelated molecules. The autoantigens recognized by NAb include DNA, tubulin, myosin, actin, cytochrome-c, transferrin, red blood cells (RBC), β -galactose (Avrameas, 1991). Detection of the reaction with more than one of these autoantigens is a marker for identifying polyreactive NAb. Secretory natural IgA has also been found to react with actin, myosin and tubulin (Quan *et al.*, 1997). NAb has also been found to recognize cholesterol (Alving and Wassef, 1999), CD4 (Hurez *et al.*, 1994), CD5 (Vassilev *et al.*, 1993), T cell receptor (TCR) (Marchalonis *et al.*, 1992), and HLA class I (Kaveri *et al.*, 1996).

The secretion of IgG1 and IgG2b isotypes of NAb is greatly influenced by T cells. NABs of these isotypes are thought to be due to their binding with autoantigens (Malanchere *et al.*, 1995). The concentration of natural IgG1 and IgG2b in BALB/c is 30-40 folds higher than in athymic mice. The deficiency in IgG production can be rapidly

reconstituted during 1-2 weeks by T cell transfer (Malanchere *et al.*, 1995). In contrast, T cells have little effect on the production of IgM in NAb. IgM levels in BALB/c mice are similar to those in athymic BALB/c nude mice (Malanchere *et al.*, 1995). In addition, natural monoclonal IgG and IgM, which are produced from non-immunized spleen cells, display similar antigen recognition repertoires (Adib-Conquy *et al.*, 1993; Mouthon *et al.*, 1995).

From newborns to adults, multireactive B cell clones exhibit newly emergent repertoires of NAb that are negatively selected during the cell maturation from bone marrow to periphery (Grandien *et al.*, 1994). Therefore, polyreactive repertoires of NAb in the circulation represent NAb-producing B cells that have escaped deletion or inactivation (Grandien *et al.*, 1994). In contrast to IgG repertoires that react with foreign antigens and show increased diversification with aging, the repertoire of self-reactive natural IgG has been found to be stable throughout life (Lacroix-Desmazes *et al.*, 1995).

1.2.2. Evidence of NAb activity against tumors.

Natural antibody has been considered as the first line of defense against viral and bacterial infections. NAb can prevent viral and bacteria dissemination to vital organs (Ochsenbein *et al.*, 1999). Natural antibody does participate in host-mediated natural resistance against neoplasia. T-cell deficient nude mice appear not to be totally immunodeficient. They do not exhibit a higher incidence of spontaneous tumors compared with normal mice and they are not more susceptible to chemical carcinogenesis

(Stutman *et al.*, 1978). A poorly tumorigenic human melanoma cell line HMCL only grows in 25% of nude mice (Jacobovich *et al.*, 1985). Although NK cells are considered to play an important role in the natural anti-tumor mechanism, treatment with sub-lethal irradiation and anti-asialo-GM1 anti-serum to abrogate NK cells does not induce an increase in tumor frequency, indicating that some other factors also contribute to natural anti-tumor resistance (Jacobovich *et al.*, 1985). Although removal of macrophages by silica-treatment significantly increased the tumor incidence of poorly tumorigenic HMCLs, some mice still remained tumor free, strongly suggesting the existence of other natural defense mechanisms such as anti-tumor NAb (Jacobovich *et al.*, 1985).

i). The relationship of tumorigenicity and NAb binding.

The early studies in our laboratory showed that the levels of NAb binding were inversely proportional to tumorigenicity (Chow *et al.*, 1981b). Pretreatment of P815-16 mastocytoma cells with syngeneic normal DBA/2 serum significantly reduced the tumorigenicity compared with untreated tumor cells (Chow *et al.*, 1981a).

It has been shown that xid B cell deficient CBA/N mice had a higher tumor incidence than normal CBA/J mice after inoculation with RI-28, a syngeneic radiation-induced T cell leukemia (Chow and Bennet, 1989; Bennet and Chow, 1991). Reconstitution with whole normal syngeneic serum NAb, ammonium sulfate-precipitated fraction or purified IgG and IgM significantly reduced the tumor frequency and latency (Chow, 1995).

L5178Y-F9 is a NK resistant T lymphoma cell line. Tumors selected from *in vivo* passage including subcutaneous (*s.c.*), intraperitoneal (*i.p.*) and intravenous (*i.v.*) inoculation of L5178Y-F9 or SL2-5 lymphomas into syngeneic mice showed increased tumorigenicity and decreased NAb binding (Chow, 1984). They also consistently exhibited reduced sensitivity to complement-mediated lysis by NAb (Chow, 1984), to natural resistance (NR) measured by ^{131}I UdR-labelled tumor elimination assay (Brown and Chow, 1985), and hypotonic lysis (Brown *et al.*, 1986).

A variant of the L5178Y-F9 selected through growth from a threshold *s.c.* inoculum in syngeneic DBA/2 strain mice exhibited a higher *in vivo* tumor frequency and liver metastasis potential (Chow *et al.*, 1983) compared with L5178Y-F9 maintained in standard tissue culture. They also displayed reduced *i.p.* clearance, suggesting that *in vivo* selection generated variants with reduced sensitivity to natural resistance (Chow *et al.*, 1983). In contrast, fluorescence-activated cell sorting (FACS) was used to isolate populations with high natural IgM antibody binding from the parental L5178Y-F9 (Tough and Chow, 1988). These high NAb binding variants consistently exhibited low tumorigenicity. Therefore, NAb binding is directly correlated with the tumorigenicity.

ii). Cell transformation and NAb binding

In the early oncogenesis, the transformed cells can express oncogene and altered gene products (Shibata, *et al.*, 1997; Bohn, 1999). A first line of defense mechanism should be able to recognize the expression of these gene products, such as the ras

oncogene. Chow's study showed that the introduction of either v-H-ras or T24-H-ras oncogene into 10T1/2 fibroblasts resulted in a significant increase in syngeneic C3H NAb binding (Tough and Chow, 1991; Tough *et al.*, 1995). In addition, variants selected through *in vivo s.c.* inoculation exhibited reduced NAb binding (Tough *et al.*, 1995). An increase in ras protein p21 by addition of ZnSO₄ to Zn⁺⁺-inducible ras-bearing 10T1/2 HI cells was also associated with significantly increased NAb binding (Tough *et al.*, 1992). *In vivo* experiments also showed that repeated *i.v.* administration of whole-serum NAb prior to tumor inoculation reduced the incidence of early tumor growth following *s.c.* injection of Zn(++)-inducible ras transfected 2HI cells into Zn(++)-treated syngeneic C3H/HeN mice (Tough *et al.*, 1992).

1.2.3. Human natural antibody and its potential role in cancer treatment

Tumor-associated antigens can elicit the production of tumor-specific antibodies (Sahin *et al.*, 1995; Chen *et al.*, 1997). In humans, the levels of polyspecific NAb including IgG, IgA and IgM are also increased in sera of patients with malignant Non-Hodgkin's lymphoma (NHL) compared with healthy controls (Kouritis *et al.*, 1994). Bohn reported that *in vitro* incubation of human cancer cell lines, such as human colon carcinoma, Colo205, small cell lung cancer and T cell lymphoma cell lines, in the presence of the purified natural human IgM antibodies resulted in growth inhibition and complement-mediated cell lysis (Bohn *et al.*, 1994).

There is abundant evidence of the existence of NAb against human neuroblastoma

(NB), the most common extracranial solid neoplasm seen in young children. Bolande and Mayer reported that the sera from pregnant women contained anti-NB IgM with cytotoxic activity (Bolande and Mayer, 1990). Ollert's study showed that approximately one third of tested normal adult sera revealed a considerable amount of natural IgM that had strong and specific cytotoxicity against human NB, whereas the sera of 11 human NB patients with active disease did not have detectable anti-NB IgM antibody (Ollert *et al.*, 1996). Fukuda reported that high levels of natural anti-NB antibody also existed in healthy Japanese children and patients with nonmalignant surgical diseases, but not in patients with stage 4 NB (Fukuda *et al.*, 1999).

The specific cytotoxicity of NAb against NB cell lines *in vitro* was in the range of 40% to 95% (Ollert *et al.*, 1996). No cytotoxic reaction was detected against several other tumor cell lines. An *in vivo* study showed that five consecutive *i.v.* injections of purified cytotoxic IgM into nude rats bearing *s.c.* human NB, completely arrested the tumor growth (David *et al.*, 1996). Activation of perivascular complement and induction of neutrophil granulocyte accumulation were considered to contribute to the cytotoxic activities that occurred 24 hr after injection of NAb (David *et al.*, 1996; Ollert *et al.*, 1996; Ollert *et al.*, 1997). Natural anti-NB IgM administration also completely inhibited tumor formation and metastasis when the antibody was injected *i.v.* simultaneously with the tumor cell injection (Engler *et al.*, 2001). It inhibited tumor growth by 90% when antibody treatment was initiated 6 days after *i.v.* tumor injection (Engler *et al.*, 2001).

Another cancer-associated molecule, MUC1, which is a heavily glycosylated protein found on a variety of glandular epithelial cells, was also found increasingly

expressed on most carcinomas, such as breast, lung, prostate, ovary and colon cancer (Peat *et al.*, 1992; Ho *et al.*, 1993). The increased incidence of MUC1 expression is correlated with a higher metastasis and poor prognosis (Hiraga *et al.*, 1998). Anti-MUC1 IgG was detected in a number of patients with ovarian, breast and non-small cell lung cancers (Gourevitch *et al.*, 1995; Rughetti *et al.*, 1993; Hirasawa *et al.*, 2000). Interestingly, natural anti-MUC1 IgM or IgG was also present in the sera of normal individuals, which exhibited significantly higher levels than patients with ovarian and non-small cell lung cancer (Richards *et al.*, 1998; Hirasawa *et al.*, 2000). Higher levels of natural anti-MUC1 antibody were strongly correlated with higher survival rates (Richards *et al.*, 1998; Hirasawa *et al.*, 2000).

1.2.4. Intravenous immunoglobulin (IVIg): the clinical application of NAb.

Intravenous immunoglobulin or IVIg is the antibody purified from pools of sera collected from many individual adult donors. It contains a rich representation of antibodies to both "self" and "non-self" proteins (Sundblad *et al.*, 1994). Since purified IgG exhibits significant inter-individual differences in terms of intensity and the patterns of immunoreactivities (Berneman *et al.*, 1993; Mouthon *et al.*, 1995), pooled IgG preparations contain a wider range of antibody repertoires that provide superior regulatory capacities than IgG from a single individual (Nobrega *et al.*, 1993).

IVIg was first applied as a supplement in the treatment of patients with agammaglobulinemia. Now, it has been used in the treatment of a number of

autoimmune disorders, such as Myasthenia gravis, Kawasaki's disease, and multiple sclerosis, etc (Kazatchkine and Kaveri, 2001). It was also reported to bind to tumors of different origins, such as melanoma (Shoenfeld *et al.*, 2001), colon carcinoma, breast carcinoma and squamous cell carcinoma of the lung (Bar-Dayyan *et al.*, 1999). Administration of IVIg inhibited the growth of SK-28 human melanoma cells in SCID mice (Shoenfeld and Fishman, 1999). Treatment with IVIg reduced the total number of peripheral lymphocytes in the patients with chronic lymphocytic leukemia (CLL) and induced the regression of Kaposi's sarcoma in an HIV patient (Bar-Dayyan *et al.*, 1999).

Despite the widespread clinical use of IVIg, little is known about its mechanism of action. IVIg inhibits the production of inflammatory cytokines such as IL-1, TNF and IL-6 that are secreted by lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) or macrophages (Shimozato *et al.*, 1990; Abe *et al.*, 1994). Pooled IVIg can inhibit the T cell response to different staphylococcal superantigens and the production of IL-6 and TNF- γ cytokines by specific binding to toxins (Takei *et al.*, 1993; Toungouz *et al.*, 1995). High doses of IVIg have also been found to inhibit the activity of NK cells (Finberg *et al.*, 1992) and the proliferation of antigen-specific T cells (Aktas *et al.*, 2001) in patients with Kawasaki syndrome.

IVIg may also prevent graft rejection and graft-versus-host disease (GVHD). Addition of IVIg to mixed lymphocyte reaction (MLR) *in vitro* resulted in the inhibition of the production of IFN- γ , TNF- α and lymphocyte proliferation, which can be reversed by recombinant IFN- γ (Toungouz *et al.*, 1996). Normal homologous and autologous human IgG, but not IgM is capable of dramatically inhibiting the proliferative response of

human PBMC to PHA-activated autologous T cells. The autologous IgG absorbed by PHA-activated T cells loses 30% of its inhibitory activity, whereas, serum absorbed by non-activated PBMC retains its activity, suggesting the inhibition is mediated by a direct effect on stimulator cells but not responder cells (Wolf-Levin *et al.*, 1993).

1.2.5. Mechanisms of NAb mediated tumor resistance.

The biological mechanism of NAb in anti-tumor resistance remains to be elucidated. It has been considered that NAb functions through activation of the classical complement pathway (Ollert *et al.*, 1996). Human IVIg has been found to bind to different tumors of epithelial origin, especially colon carcinoma, breast carcinoma and squamous cell carcinoma of the lung and induce antibody dependent cell-mediated cytotoxicity (ADCC) response against tumors (Bar-Dayyan *et al.*, 1999). Human IVIg may also stimulate the production of IL-12 and enhance NK cell activity *in vitro* (Shoenfeld and Fishman, 1999).

Researchers are increasingly interested in the role of NAb binding molecules in anti-tumor resistance. NAb has been found to induce apoptosis of human NB cells *in vitro* and *in vivo* (David *et al.*, 1999). A 260Kd molecule, NB-p260 was found to be highly associated with the NAb-mediated apoptosis (Ollert *et al.*, 1996; David *et al.*, 1999). It was specifically detected on human NB cells and not on various normal cells, melanoma, osteosarcoma, lymphoma and some carcinomas. NAb-induced apoptosis was abolished by either preincubation of anti-NB IgM with immobilized purified NB-p260 or

preincubation of murine anti-NBp260 IgG with NB cells (David *et al.*, 1999).

The preliminary data in our lab showed that NAb binding might be correlated with CD45RA expression on L5178Y-F9 and its high NAb binding variants (Chow *et al.*, 1999). Considering that CD45 comprises 10% of cell surface molecules, this observation suggests that CD45RA may be a target for NAb.

1.3. CD45

CD45, also called the leukocyte common antigen (LCA), T200, B220 and Ly5, is a highly glycosylated transmembrane protein exclusively present on all hematopoietic cells except erythrocytes and their progenitors. Because of the alternative splicing of variable exons 4, 5, and 6 within the mRNA, six to eight different isoforms of MW 180-240 are produced. CD45RA, CD45RB and CD45RC are the products of the individual variable exons 4, 5, and 6 respectively. Other isoforms include CD45RAB, RAC, RBC, and RABC. If none of the three variable exons is involved in the protein synthesis, the product is called CD45RO (Trowbridge and Thomas, 1994). Pan-CD45 specific antibodies react with a common portion of all CD45 molecules. The extracellular portion of CD45 is a heavily glycosylated, cysteine-rich segment which ranges from about 400 to 550 amino acids (AA), and the transmembrane segment contains 22 AA (Clark and Ledbetter, 1989). Both N- and O-linked glycosylation sites have been identified in the extracellular segment and they exhibit dramatic alterations with changes in the expression of CD45 isoforms (Thomas, 1989). The amino-terminal region of approximately 200 AA

is the site of O-linked glycosylation which contains the serine/threonine rich domain and the variable portion of variable CD45 exons. Following this domain are two cysteine domains with 100 and 220 AA (Thomas, 1989).

1.3.1. CD45, PTPase and Src tyrosine kinase

CD45 has two intracellular domains which possess protein tyrosine phosphatase (PTPase) activity (Tonks *et al.*, 1990), activating or inhibiting Src family kinase activity. The Src-family is a group of intracellular tyrosine kinases. Its N-terminus interacts with cell membranes through lipid modification and amino acids (Thomas and Brown, 1999).

CD45 can activate the protein tyrosine kinase (PTKase) P56^{lck} and P59^{fyn} by dephosphorylating negatively regulatory residues Tyr-505 and Tyr-531 respectively (Mustelin *et al.*, 1989; Mustelin and Altman, 1990; Ostergaard *et al.*, 1989; Ostergaard and Trowbridge, 1990; Hurley, 1993). The recent study by Ashwell and Baker showed that CD45 also inhibited the PTKase P56^{lck} by dephosphorylating the positively regulatory residue Tyr-394 (Ashwell and D'Oro, 1999; Baker *et al.*, 2000). Both P56^{lck} and p59^{fyn} are PTKases that are tightly associated with CD4 (or CD8) and TCR/CD3, respectively (Rudd *et al.*, 1988; Koretzk *et al.*, 1993). Tyrosine phosphorylation of certain intracellular proteins plays an important role in the regulation of the growth of normal and malignant cells (Cantley *et al.*, 1991). It is determined by a dynamic equilibrium between two opposing reactions: PTKase-mediated phosphorylation and PTPase-induced dephosphorylation (Hunter, 1987). More than 90% of membrane-bound

PTPase activity is due to CD45 (Mustelin *et al.*, 1989). These two PTPase domains and the membrane proximal region are necessary and sufficient for PTPase activity and TCR signal transduction (Volarevic *et al.*, 1993; Desai *et al.*, 1994).

1.3.2. CD45 expression on T and B cells

The expression of different CD45 isoforms is associated with the state of maturation and activation of both T and B lymphocytes (Thomas, 1989). The PTPase activity of CD45 is required in the maturation of both T cells and B cells (Ong *et al.*, 1994; Benatar *et al.*, 1996; Seavitt *et al.*, 1999). Kung reported on a patient with CD45 deficiency due to mutations which resulted in a severe combined immunodeficient disease (Kung *et al.*, 2000). CD45RO expression regulates the efficacy of TCR-derived signals that induce apoptosis of double positive thymocytes (Fujii *et al.*, 1992; Ong *et al.*, 1994). In the periphery, T cell activation is accompanied by the loss of the high MW isoform of CD45RA and an increase of the low MW CD45RO isoform (Akber *et al.*, 1988; Yamada *et al.*, 1990). CD45RA expression is rapidly increased in the early stage (24h) of human T cell activation, and CD45RO expression starts to increase 48 hours after activation (Yamada *et al.*, 1990, 1992). Similarly, naive and memory T cells are CD45RC⁺ and CD45RC⁻, respectively (Zapata *et al.*, 1994). The shift from CD45RC⁺ to CD45RC⁻ with antigen stimulation is not unidirectional and irreversible. Both *in vitro* and *in vivo* studies show that CD45RC⁻ T cells can switch back to CD45RC⁺ cells in the absence of allogeneic stimulation (Sarawar *et al.*, 1993; Bunce and Bell, 1997). The re-

expression of the high MW CD45RC isoform also causes functional restoration of naïve T cells (Sarawar *et al.*, 1993).

The expression of CD45RB and CD45RA is independently regulated during activation of human CD4⁺ naïve T cells (Horgan *et al.*, 1994). Although resting tonsil B cells express high MW isoforms of CD45R, unlike T cells, the activation of B cells only induces minor changes in the expression of CD45R and CD45RO (Zola *et al.*, 1990). Additionally, the expression of CD45RO on B cells is not a memory phenotype but rather represents a late stage in the differentiation to plasma cells (Jensen *et al.*, 1991b; Jensen *et al.*, 1992).

1.3.3. Glycosylation of CD45

CD45 is heavily glycosylated, especially with O-linked carbohydrates (Pulido and Sanchez, 1989; Sato *et al.*, 1993). The function of CD45 also depends on its glycosylation status. The glycosylation of CD45 on the thymocytes is associated with the maturation and development of thymocytes (Uemira *et al.*, 1996; Baldwin and Ostergaard, 2001). The glycosylation pattern of CD45 can be changed by the proliferative status in tissue culture. The stationary lymphocytes in 5-day culture exhibit less N-glycosylation than exponentially growing cells, resulting in mobility shift in SDS-PAGE (Ohta *et al.*, 1994).

The intact glycosylation of CD45 is required for stability and proper transport towards the cell membrane and maintenance of PTPase (Pulido and Sanchez, 1992).

Incubation of the N-glycosylation inhibitor tunicamycin with K562 cells markedly inhibits CD45 surface expression and PTPase activity without interrupting protein synthesis of CD45 (Pulido and Sanchez, 1992).

Sialylation of CD45 is related to the responsiveness of T cells to IL-2. Brutkiewicz has reported that an anti-mouse CD45RB mAb CZ-1 recognizing a sialic acid-dependent epitope can be used to distinguish IL-2-responsive from non-responsive subpopulations. Only CZ-1⁺ but not CZ-1⁻ cells respond strongly to IL-2 (Brutkiewicz *et al.*, 1993). Sialylation of CD45 is also associated with B cell proliferation. Unlike other anti-CD45 mAbs that inhibit anti-Ig-induced B cell proliferation, an anti-sialic acid CD45 mAb, 136-4B5 used in Alsinet's study enhances B cell proliferation mediated by anti-Ig (Alsinet, 1990).

Lazarovits has also reported that anti-CD45RB mAb MT3 that recognizes terminal sialic acid can inhibit allogeneic MLR on day 6. MT3 markedly inhibits the expression of the IL-2 receptor on CD4⁺ T cells and blocks CD4⁺CD45RA⁻ cells entering the proliferative phase (Lazarovits *et al.*, 1992). Two injections of another anti-CD45RB mAb MB23G2, which also reacts with terminal sialic acid prior to or after transplantation, prevent renal allograft rejection (Lazarovits *et al.*, 1996). MB23G3 could induce tolerance of peripheral lymphocytes by enhancing the PTPase activity of CD45 (Lazarovits *et al.*, 1999).

1.3.4. CD45 associated molecules.

CD45 has been found to be associated with many cell surface molecules, such as CD2, CD3, CD4 and CD8 *etc.* CD45 is required in the signaling mediated by either TCR, anti-CD2, anti-CD3 or anti-CD4 (Koretzky *et al.*, 1990, 1991; Deans *et al.*, 1992). CD45 is associated with mIgM on B cells (Justement *et al.*, 1991) and it is also required for mIg-induced signal transduction and B cell growth (Ogimoto *et al.*, 1995).

The association of CD4 with CD45 is dependent on the specific external domain of various CD45 isoforms, not requiring its cytoplasmic domains. CD4/TCR complex preferentially interacts with low MW CD45 isoforms, which exhibit much higher responses to peptide/APC stimulation (Dianzani *et al.*, 1990; Leitenberg *et al.*, 1996). The expression of CD45 and their PTPase domains are required for ligand-induced TCR downregulation (Kastrup *et al.*, 2000).

The natural ligand of CD45 is CD22 (SgROI *et al.*, 1995), a B cell specific receptor of the immunoglobulin superfamily. CD22 is a sialic acid-binding I-type lectin and recognizes Sia α 2-6Gal β 1-4GlcNAc on N-linked oligosaccharides of the CD45RO isoform (Powell *et al.*, 1995). Cross-linking of CD45 with anti-CD45 mAb separates CD45 from CD22, resulting in an increase in tyrosine phosphorylation of CD22 (Greer and Justement, 1999).

Studies using human tumor infiltrating lymphocytes (TIL) show that CD28 is associated with CD45RO. Both anti-CD28 and anti-CD45RO mAbs co-precipitate 180 KD CD45RO and 90 KD CD28 molecules. Functionally, only CD45RO⁺ TILs are

responsive to anti-CD28 mediated proliferation (Zocchi *et al.*, 1992).

CD45 associate-protein, CD45-AP is a 30KD lymphoid-specific protein with a short extracellular domain of 7 amino acids, a transmembrane region and a cytoplasmic domain of 150 amino acids. It is strongly associated with both the CD45 monomer and dimer directly through the transmembrane region (Cahir McFarland and Thomas, 1995). Although no such protein can be detected in the CD45-deficient T cell clone, CD45-AP mRNA is present and CD45AP is retained in the ER, suggesting that the association starts within ER (Cahir McFarland *et al.*, 1997). However, Kung's study showed that CD45AP was not crucial in the regulation of CD45-mediated Src-family kinase activity (Kung *et al.*, 1999).

CD7, a 40KD glycopolypeptide expressed on the majority of human peripheral T cells has been found to be associated with CD45. A CD7 immunoprecipitate has protein tyrosine kinase activity and CD7 cross-linking on Jurkat T cells causes tyrosine phosphorylation of CD45 (Lazarovits *et al.*, 1994).

1.3.5. CD45 expression on leukemia cells

T-acute lymphoblastic leukemia (T-ALL) and B-chronic lymphoblastic leukemia (B-CLL) predominantly express the memory phenotype CD45RA⁺RO⁺ (Falcao and Garcia, 1993; Yu *et al.*, 2000). In contrast, B-origin ALL and B cell non-Hodgkin's lymphoma (NHL) are characterized by high expression of CD45RA or CD45RABC and very low or virtual absence of CD45RO (Caldwell *et al.*, 1991; Schiavone *et al.*, 1995;

Yu *et al.*, 2000). However, other malignant B cell populations display the phenotype of terminal differentiation, increased CD45RO and decreased CD45RA expression, such as Waldenström's Macroglobulinemia (Jensen *et al.*, 1991a and 1991b) and multiple myeloma (MM) (Jensen *et al.*, 1992).

1.3.6. CD45 and tumor transformation

Many neoplastic cells display an abnormal tyrosine kinase that induces alteration of the balance between PTPase and PTKase, profoundly affecting cell proliferation. Baker's study showed that CD45 suppressed the tumorigenic potential of the lck kinase by dephosphorylation of the Tyr394, an autophosphorylation site (Baker *et al.*, 2000). In CD45 deficient mice, the lck kinase of thymocytes exhibited hyperactivity, leading to increased resistance to apoptosis and resulting in the development of aggressive thymic lymphoma (Baker *et al.*, 2000). Vanadate, a potent inhibitor of PTPase can cause normal cells to express a transformed phenotype (Klarlund, 1985).

1.3.7. CD45 and apoptosis

Cross-linking of CD3 and CD45 or CD45 alone can induce apoptosis in both T and B cell lines from various developmental stages (Klaus *et al.*, 1996). Both anti-CD45RA and anti-panCD45 mAbs cause T cell death that requires phosphatase activity of CD45. Apoptosis was abrogated by addition of PTPase inhibitors sodium o-vanadate

and phenylarsine oxide (Klaus *et al.*, 1996). CD45-mediated apoptosis has been considered to be independent of Fas (CD95, APO-1) ligation (Latinis and Koretzky, 1996). CD95-mediated apoptosis does not depend on the expression of either p56^{lck} or CD45. Anti-APO-1 can trigger apoptosis of CD45 or p56^{lck} deficient Jurkat mutants, as well as that of their wild type counterparts (Schraven and Peter, 1995).

The apoptosis mediated by both anti-CD95 and anti-CD45 is very rapid and displays maximal effects within 6-8 hr. However, unlike anti-CD95 or anti-TCR induced programmed cell death which is characterized by cytoplasmic and nuclear condensation and DNA fragmentation, CD45 mediated cell death lacks double stranded DNA cleavage into oligonucleosomes (Klaus *et al.*, 1996).

Activation of peripheral T cells by PMA induces the expression of functional Fas ligand that contributes to increased susceptibility of activated T cells to apoptosis by monocytes (Wu *et al.*, 1996). Anti-Fas, anti-CD11a, anti-CD18, or anti-CD45RA mAbs could prevent the cell death triggered by PMA plus monocytes. Furthermore, the apoptotic process did not occur in a CD45-deficient variant of Jurkat cells (Wu *et al.* 1996). The apoptotic response was recovered by transfection with cDNA encoding CD45RA, suggesting that CD45RA participated in the death of peripheral T cells (Wu *et al.* 1996)

Galectin-1, a beta-galactoside-binding protein expressed on endothelial cells induces apoptosis of activated T cells via CD45 (Perillo *et al.*, 1995). It was considered the natural ligand of CD45 (Fouillit *et al.*, 2000). Exposure of activated T cells or T leukaemic cell lines to purified galectin-1 or endothelial cells for 30 min induced an

irreversible apoptosis of T cells. The glycosylation status of CD45 plays an important role in galectin-1-induced T cell death. N-glycans of pan-CD45 may be involved in the galectin-1-mediated cell death, whereas, O-glycans of high MW CD45 isoforms may regulate the effect of galectin-1 by masking N-glycan (Perillo *et al.*, 1995). Oligosaccharide can mediate the clustering of CD45, facilitating galectin-1-induced cell death (Nguyen *et al.*, 2001). The apoptosis is attributed to the decreased membrane-associated PTPase activity of CD45 by galectin-1 (Walzel *et al.*, 1999).

1.3.8. The role of CD45 in CTL and NK cell killing.

CD45 plays a critical role in NK and CTL-mediated killing. CD45 negative variants of RNK-16, a rat leukemia line with NK activity, fail to kill YAC-1 and RL-male-1 tumor target cells (Bell *et al.*, 1993). CD45 on NK3.3 cells is also physically associated with p56^{lck} but not with p59^{lyn} (Xu and Chong, 1995). The cell-mediated cytotoxicity (CMC) of CTL and NK cells depends upon phosphatase activity. The addition of phosphatase inhibitor Calyculin A markedly suppressed NK-CMC and CTL-CMC by 80-90%. Calyculin A also inhibits NK induced ADCC by 70-90%. The defect of PTPase can be completely restored by the addition of IL-2 (Bajpai and Brahmi, 1994).

The cytolytic activity of NK cells can be inhibited by blocking of adhesion between effector and target cells mediated by LFA1 α or LFA β molecules which are functionally associated with CD45 on effector cells (Poggi *et al.*, 1993). Hanaoka's study showed that an anti-CD45RO mAb suppressed the apoptosis of mouse T lymphoma CS-

21 cells by inhibiting their adhesion to CA-12 stromal cells (Hanaoka *et al.*, 1995).

1.3.9. Anti-CD45 and cancer therapy

Because CD45 is expressed by most leukocytes and their precursors, anti-CD45 mAb has been successfully used to selectively deliver much higher levels of radiation to lymphohematopoietic tissues, such as spleen, bone marrow and lymph nodes to treat acute myeloid leukemia (AML)/myelodysplastic syndrome (MDS) and ALL (Matthews *et al.*, 1992, 1999). The treatment regimen includes administration of therapeutic dose of ¹³¹I-labeled anti-CD45 plus cyclophosphamide to ablate bone marrow, followed by bone marrow transplantation (Matthews *et al.*, 1992, 1999). Seven of 25 patients with AML/MDS are surviving disease-free 26-100 months and three of 9 patients with ALL are surviving disease-free 34-82 months post-transplant (Matthews *et al.*, 1999; Ruffner and Matthews, 2000). The studies by Kroon showed that *i.v.* administration of anti-CD45 mAb for 4 days effectively eliminated systematic dissemination of *i.v.* or *s.c.* inoculated human non-Hodgkin's lymphoma B cell lines DoHH2 and BEVA in severe combined immunodeficient (SCID) mice (de Kroon *et al.*, 1996).

1.4. NAb, CD45 AND AUTOIMMUNE DISEASES

Autoantibodies have been associated with many autoimmune diseases. The increased level or altered reactivity of NAb in the circulation is regarded as one of the

major mechanisms in the induction of autoimmune diseases (Naparstek and Plotz, 1993). Several diseases are associated with increased levels of pathologic autoantibodies, such as anti-acetylcholine receptor antibodies in myasthenia gravis (Schonbeck *et al.*, 1990), anti-thyroid stimulating hormone (TSH) in hyperthyroidism (Burman and Baker, 1985), anti-IgM autoantibodies (rheumatoid factors, RF; Carayannopoulos *et al.*, 2000) and anti-TCR in rheumatoid arthritis (RA; Robey *et al.*, 2000).

The mechanisms of the development of autoimmune diseases are still unknown. The abnormal distribution of CD45RO⁺ activated T cells was found in various autoimmune diseases. CD4⁺CD45RO⁺ T cells were predominantly present in the peripheral blood and synovial fluid from patients with RA (Mamoune *et al.*, 2000a). They were also found increased in the peripheral blood and cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS; Barreau *et al.*, 2000). Peterson reported that recently activated CD45RA⁺RO⁺ T cells and CD45RO⁺ memory cells were primarily detected in the newly diagnosed diabetic patients and those with longer disease duration, respectively (Peterson *et al.*, 1999). In addition, the PTPase activity of CD45 was lower in unstimulated T cells of SLE patients (Takeuchi *et al.*, 1997; Blasini *et al.*, 1998). Recent studies showed that point mutation of the gene encoding CD45 caused altered expression of isoforms that could induce polyclonal lymphocyte proliferation and the production of autoantibodies, resulting in autoimmune disease, such as nephritis (Majeti *et al.*, 2000) and MS (Jacobsen *et al.*, 2000).

The existence of anti-lymphocyte NAb may be associated with some autoimmune diseases, such as SLE (Osman and Swaak, 1994). Sera from SLE patients have

significantly higher levels of anti-lymphocyte autoantibodies (IgM). The presence of these antibodies is associated with a state of lymphocyte activity in these patients. (Czyzyk *et al.*, 1996). Mimura's study also showed that the elimination of CD4⁺CD45RA⁺ T cells might be attributed to anti-CD45 IgM autoantibodies reactive with high MW isoform of CD45RA, but not CD45RO (Mimura *et al.*, 1990a, 1990b). Twenty-five percent of sera from SLE patients contained both IgG and IgM, which reacted with the asialylated sugar portion of purified CD45 (Mamoune *et al.*, 1998; Fernsten *et al.*, 1994). Additionally, the most recent study showed that anti-CD45 antibodies against both low MW and high MW isoforms were detected from the sera of most SLE patients (Mamoune *et al.*, 2000b). An anti-CD45RB mAb was also used to treat experimental allergic encephalomyelitis (EAE). Anti-CD45RB antibody treatment prevented the development of EAE in mice immunized with myelin basic protein by altering T cell proliferation and cytokine production (Schiffenbauer *et al.*, 1998).

The extensive studies have shown that both NAb and CD45 play important roles in the anti-tumor resistance and cell regulation. There is no evidence to show whether or not NAb acts through CD45 in the anti-tumor resistance and the regulation of lymphocyte activation. This study is 1) to examine the relationships between NAb binding and the expression of different CD45 isoforms, pan CD45, CD45RA, RB and RC, 2) to identify the correlation between CD45 expression and tumorigenicity, and 3) to assess whether NAb binds directly to CD45 on the cell surface.

2. MATERIALS AND METHODS

2.1. MICE AND SERA.

DBA/2 mice (9-12 weeks old) were obtained from the University of Manitoba Vivarium. A/J mice (8-12 weeks old) were obtained from Jackson Laboratory, Bar Harbor, Maine. Normal mice were bled from the axilla. After the blood was clotted at 4°C, the serum was isolated by centrifugation and stored at -20°C. The blood from at least 30 mice was mixed and the serum was collected as a batch. Several batches were used during the study.

2.2. TUMOR CELL LINES.

L5178Y is a spontaneous T lymphoma of DBA/2 mice maintained in our laboratory for many years. L5178Y-F9 was derived from the L5178Y through two successive clonings using a sloppy agar procedure (Chow *et al.*, 1980). LYNAb⁺ was derived from L5178Y-F9 cells through three successive cycles of treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) and FACS for the 3% highest NAb binders (previously named L5178Y-F9 TPA/NAb⁺3 by Tough *et al.*, 1988). These cell lines were maintained in Fisher's Medium (GIBCO) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin (GIBCO).

YAC wild type (YAC-WT) is a Moloney virus-induced NK sensitive T

lymphoma passaged in A strain mice (Klein and Klein, 1964). It was cultured in 9% FBS-DMEM. YAC-N1 (kindly provided by Dr. Ostergaard) was a CD45-deficient variant of YAC-WT and generated by FACS sorting. The YAC-N1 expressed barely detectable CD45 mRNA without any CD45 associated tyrosine phosphatase activity (Volarevic et al 1992). YAC-33 (provided by Dr. Ostergaard) was produced by transfection of CD45RABC cDNA into YAC-N1 cells.

2.3. REAGENTS AND CULTURE MEDIUM.

Reagents used in this study include R-phycoerythrin (PE) conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA), neuraminidase (Type V, Sigma, St. Louis, MO, USA), N-hydroxysuccinimido-biotin (NHS-biotin) (Sigma), CNBR-activated Sepharose 4B (Sigma), Protein A (Pharmacia Biotech, Uppsala, Sweden), peroxidase conjugate avidin-horseradish (Bio-Rad Laboratories, Hercules, CA), protein-G Sepharose-4 fast flow (Pharmacia), enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham International plc. Buckinghamshire, England), Dimethylsulfoxide (DMSO) and R-PE conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania).

The cell culture media related materials included Dulbecco's Modified Eagle Medium (DMEM, high glucose, with L-glutamine, without sodium pyruvate), Fischer's Medium (FM, with L-glutamine), RPMI 1640, FBS and Hepes (GIBCO BRL, Life Technologies, Grand Island, NY).

2.4. ANTIBODIES.

Antibodies used in this study included FITC conjugated goat anti-mouse IgG (whole molecule; Sigma), FITC-conjugated goat anti-mouse IgM (μ -chain specific; Sigma), FITC conjugated goat anti-rat IgG (whole molecule; Sigma), rabbit anti-mouse CD45 serum (provided by Dr. Ostergaard, H., University of Alberta), horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Sigma), anti-CD44 mAb IM-7 (PharMingen, San Diego, CA), anti-CD45 mAb DNL-1.9 anti-CD45RC IgG_{2a} (PharMingen, San Diego, CA; Dessner *et al.*, 1981), and biotin labeled anti-CD45RA mAb 14.8 (PharMingen, San Diego, CA).

2.5. HYBRIDOMAS.

We cultured several hybridoma cell lines to produce rat anti-mouse CD45 and CD45R mAbs. They included M1/89.18.7 HK anti-CD45 IgG_{2b} (TIB124, American Tissue Type Collection, ATCC, Rockville MA; Springer *et al.*, 1978) and I3/2 anti-CD45 IgG_{2b} (kindly provided by Dr. H. L. Ostergaard, University of Alberta and Dr. I. Trowbridge, Salk Inst. San Diego CA; Trowbridge *et al.*, 1978). I3/2 immunoprecipitated iodinated molecules of 190-220 KD and no other molecules, from lymphoid cells surface labelled via lactoperoxidase (Trowbridge *et al.*, 1978). M1/89.18.7 HK immunoprecipitated iodinated molecules of 180-210 KD from lactoperoxidase-labelled murine T cell hybridoma 13.13 cells, and minor bands at 150

KD were seen but not discussed (Goldman *et al.*, 1992). Hybridomas for CD45 isoform-specific and other monoclonal antibodies were 14.8 anti-CD45RA IgG_{2b} (TIB164, ATCC; Marvel *et al.*, 1989), MB23G2 anti-CD45RB IgG_{2a} (HB220, ATCC; Birkeland *et al.*, 1988), and isotype controls 2.43 anti-Lyt-2.2 IgG_{2b} (TIB210, ATCC) and Y13-238 anti-ras p21 IgG_{2a} (CRL1741, ATCC).

At the beginning, M1/89.18.7.HK, 14.8 and CRL 1741 were maintained in 10% FBS-DMEM, 2.43 in 20% FBS-DMEM, MB23G2 in 10% FBS-RPMI 1640 and I3/2 in 5% FBS-DMEM. Then they were cultured in media with 5% FBS. At the end, the cells were collected, washed with medium without FBS once and cultured in media without FBS in the presence of 25mM Hepes (GIBCO). When more than 90% of the cells died, cell suspensions were centrifuged and supernatants were collected for purification.

2.6. PURIFICATION OF MONOCLONAL ANTIBODIES.

An equal volume of saturated ammonium sulfate (SAS) was added drop by drop to FBS-free medium supernatant of hybridomas at room temperature. The mixture was stored at 4°C overnight and centrifuged at 5,000 rpm at 4°C for 30 min. The supernatant was removed and the pellet was resuspended by the addition of phosphate-buffer saline (PBS) to 1/20 of the initial volume. The precipitated suspension was dialyzed against 1 liter of PBS for 2 days with several changes of PBS. The insoluble debris was removed by centrifugation.

Two ml of protein G-Sepharose beads were loaded in a column and washed with

at least 20 ml of PBS. SAS-purified supernatant was passed through the column slowly (10-15 drops per min). After intensive washing with PBS, at least 20 ml, antibody was eluted with 0.1 M Glycine-HCl at pH 2.7. The eluate was immediately neutralized by addition of 1M Tris-HCl pH 10. The amount of protein in the eluate was measured by spectrophotometer PMQ II (Carl Zeiss, Germany) at a wavelength of 280nm. The eluates in different tubes were mixed and dialyzed against PBS as described above and an extinction coefficient of 1.4 per mg/ml. Purified monoclonal antibodies were quantitated and concentrated to >0.6 mg/ml by centrifuging through Centricon-30 tubes (Amicon, Beverly, MA). Antibodies were stored at 4°C in the presence of 0.02% sodium azide. When different mAbs were purified, the column was regenerated by washing with 1M LiCl, 2M Urea and 0.1M Glycine-HCl pH 2.7 to remove all nonspecific binding to the column.

2.7. CONJUGATION OF MONOCLONAL ANTIBODIES TO SEPHAROSE 4B.

The procedure was based on the *Affinity Chromatography* handbook of Pharmacia. Briefly, mAb was dialysed for 4 hr against the coupling buffer, 0.1 M NaHCO₃ buffer, pH 8.3 containing NaCl at 0.5 M. One gram of CNBR-activated Sepharose 4B was swollen in 200 ml HCl for 15 min in 1 mM HCl and washed twice with the coupling buffer. MAb was immediately combined with the gel suspension at the recommended ratio of one ml gel to 5-10 mg protein at 4°C and mixed overnight on a nutator. After centrifugation to remove the supernatant, blocking buffer, 0.2 M glycine

pH 8.0 was added to the gel and incubated for 2 h at room temperature to block the remaining active groups. Any free antibody was removed by 4 washes alternating between coupling buffer and 0.1 M acetate buffer containing 0.5 M NaCl at pH 4.0, followed by one wash with PBS. The amount of protein labeled on the gel was calculated by subtracting the protein leftover in the supernatant after labeling from that in the initial solution. The concentration of antibody on the gel was 1-2 mg/ml gel. MAbs-Sepharose conjugates were stored at 4°C.

2.8. COUPLING OF PROTEIN A TO SEPHAROSE 4B.

The procedure was the same as the conjugation of mAbs to CNBR-activated Sepharose 4B column except using protein A rather than mAb.

2.9. PURIFICATION OF NATURAL ANTIBODY FROM SERUM.

An equal amount of SAS was added drop by drop to DBA/2 serum at RT. The precipitate was separated by centrifugation 18 h later at 4°C. The protein was re-dissolved by the addition of a volume of PBS equivalent to the initial volume of serum, followed by dialysis against PBS for 2 days with several changes of the PBS. The insoluble material was removed by centrifugation. Five mls of Protein A-Sepharose 4B beads were put in a column and washed with at least 50 ml of PBS. The column was washed by the addition of 10 columns of coupling buffer, 1.5M Glycine-NaOH

containing 3M NaCl at pH 8.9. An equal volume of coupling buffer was added to the antibody and the solution was loaded on the top of the column. The flow rate was controlled to 10-15 drops per min. Protein content in the effluent was determined using a PMQ II spectrophotometer at 280 nm and an extinction coefficient of 1.4 per mg/ml. The column was washed with 50 ml of coupling buffer or until the O.D. reading was zero in the effluent, followed by eluting with 0.1 M acetate buffer containing 0.15M NaCl at pH 3.0. Elates were collected and immediately neutralized by the addition of 1M Tris-HCl at pH 10. Eluates were dialysed against PBS for 2 days with several changes of PBS. Finally, the solution was concentrated to the original volume. Concentrations of 0.7-1.5 mg/ml of IgG were obtained by this method.

2.10. BIOTINYLATION OF MONOCLONAL ANTIBODIES.

The procedure was based on the instruction of VECTOR Laboratories. Briefly, Biotin N-hydrosuccinimide ester was dissolved in DMSO at a concentration of 25-50 mg/ml. MAb was dialysed against 1 liter of 0.1M NaHCO₃ for 4 h at a concentration of, at least, 1.5 mg/ml. An aliquot of the biotinylating reagent equal to 1/10th of the protein was added to the antibody solution. The reaction was incubated at room temperature for 2 h. Ten mg of glycine was added to stop the reaction, followed by dialysis to remove the excess biotin. The biotinylated mAb was stored at 4°C in the presence of 0.02% NaN₃.

2.11. ANTIBODY BINDING ASSAY.

Flow cytometry was used to analyze antibody binding to the L5178Y-F9, LYNAb⁺, and its *in vivo* selected variants according to Sandstrom's procedure with some modification (Sandstrom and Chow, 1987). Pellets of 3×10^5 tumor cells were resuspended in 100 μ l aliquots of NAb or mAbs. The saturating binding concentration was $\frac{1}{4}$ diluted DBA/2 whole serum for NAb, 100 μ g/ml for purified mAb 14.8, 50 μ g/ml for MB23G2, 50 μ g/ml for DNL-1.9, 1/1000 dilution of the mAb culture supernatant for I3/2 anti-CD45 and undiluted mAb culture supernatants for M1/89.18.7 HK anti-CD45. The saturating concentration was determined by a single titration assay. All antibodies except I3/2 and M1/89.18.7 HK were diluted with wash medium consisting of FM containing 5% FBS, 10mM HEPES and 0.1% NaN₃. Two controls were employed for mAb binding, isotype and medium controls. The former was used at the same concentrations as each anti-CD45 mAb. The latter was used to set the background. Only one control was applied for NAb, the wash medium. Both NAb and monoclonal antibodies were incubated with cells for 1 hr at 4°C. Cells were agitated every 15 to 20 min during the incubation. Cells were washed once after NAb incubation with 3 ml wash medium or twice after mAb incubation with a total of 6 ml wash medium at 4°C, followed by reaction with 100 μ l of the second antibodies at 4°C for 20 min. The second antibodies for NAb were 100 μ l of a 1/15 dilution of FITC anti-mouse IgG (whole molecule specific) plus a 1/25 dilution of FITC anti-mouse IgM (μ chain specific). The second antibody for mAb was 100 μ l of a 1/64 dilution of FITC anti-rat IgG (whole

molecule specific). Finally, cells were washed once and fixed in 200 μ l of 1% paraformaldehyde. The level of NAb binding and CD45 expression on the cell surface were evaluated by flow cytometry in terms of the mean channel fluorescence (MCF) minus the control MCF.

2.12. *IN VIVO* SELECTION OF TUMORS.

High NAb binding LYNAb⁺ cells maintained in culture were washed with Hank's Balanced Salt Solution (HBSS). Aliquots of 4X10³ cells in 0.2 ml HBSS were obtained by serial dilution from the original suspension. An inoculum of 2X10³ cells was injected *s.c.* into the middle of the shaved back of each of two 8-10 week old syngeneic DBA/2 mice. The mice were sacrificed 34 days after the injection by bleeding in order to minimize red blood cell contamination in the tumor tissue during dissection. The tumor tissue was dissected aseptically from the injection site and put into a petri dish with 10% FBS-FM. The tissue was cut into very small pieces. Enough medium was added and the whole suspension was transferred into a sterile 25 ml Universal tube. After small pieces of tissue had settled, the supernatant containing mainly single cell suspension was collected. The single cell suspension named X1SC was washed twice with 10% FBS-FM and returned to the culture. After 3 days of culture, enough cells were obtained to test their NAb binding and CD45 expression. In the meantime, some cells were frozen for future use. Cells from the second mouse were collected as above and kept in the liquid nitrogen.

Thirty days later, X1SC cells were harvested from tissue culture and an aliquot of 2×10^3 was inoculated *s.c.* into each of two DBA/2 mice as described above. After 26 days growth *in vivo*, X2SC cells were isolated from the mice and returned to tissue culture. After the 9th day in tissue culture, an adequate number of cells originating from one mouse was obtained for the assays of NAb binding and CD45 expression. Cells from the other mouse were frozen. Following 11 days of culture *in vitro*, an aliquot of 2×10^3 washed X2SC cells were injected into each of three DBA/2 mice. As before, X3SC cells were isolated from *s.c.* tumors after 23 days growth *in vivo*. From the 2nd to the 5th days of culture, X3SC cells from all these three mice were examined for NAb binding and CD45 expression. NAb binding and CD45 expression of these three *in vivo* selected cell types were examined for 2 months. The data from the first 2 weeks were used for statistic analysis.

2.13. TUMORIGENICITY ASSAY.

Three cell lines were employed in this study, LYNAb⁺, X2SC and X3SC. The number of LYNAb⁺ to be injected was determined first, since LYNAb⁺ was supposed to exhibit lower tumorigenicity. Since X1SC was not expected to exhibit significantly higher tumorigenicity than LYNAb⁺, it was not used in this assay. In order to compare the tumorigenicity of different cell lines in the mice, the criterion was that at least half of the mice inoculated with LYNAb⁺ were tumor-free. The previous study showed that *s.c.* injection of 50 cells of L5178Y-F9 induced tumor growth in 2/8 of DBA/2 mice. In this

study we first tried 1000 LYNAb⁺. An aliquot of 0.1 ml containing 1000 cells was injected *s.c.* into a shaved area in the middle of the lower back for each mouse. Tumors were detected in all of the mice. Thus, 100 cells from each cell lines were used.

These three cell lines maintained in tissue culture were washed. They were serially diluted in HBSS so that an aliquot of 0.1 ml containing 100 cells could be injected. As above, each cell line was injected into a group of 6 mice. The latency was recorded, which was defined as the length of time from the date of injection to the date of the appearance of visible tumor. The tumor size was assessed as the multiple of the largest diameter and the diameter at a 90° angle measured with calipers. The final tumor frequency was assessed 2 weeks after the appearance of the last tumor and mice were then sacrificed. This assay was repeated once. Therefore, there were total 12 mice in each group.

2.14. NEURAMINIDASE TREATMENT OF L5178Y-F9.

An aliquot of 2.5×10^6 L5178Y-F9 was washed twice with PBS to remove the serum proteins. Cells were resuspended in 380 μ l of HBSS/NaOH buffer (made with 3.9 ml HBSS plus 5 μ l 0.5 M NaOH) plus 20 μ l neuraminidase (Type V from *Clostridium perfringens* 100 U/ml, Sigma), containing 2U total, or 400 μ l HBSS/NaOH as control. After incubation for 50 minutes in a 37°C water bath with shaking every 10 minutes, the reaction was terminated by the addition of 4 ml wash medium, which was identical to that used in the antibody binding assay, followed by centrifugation. NAb binding and CD45

expression were examined as described above.

2.15. BIOTINYLATION AND LYSIS OF CELLS.

Cells were biotinylated according to Meier (Meier *et al.*, 1992) with some modification. An aliquot of 1×10^7 cells was washed twice with PBS and the cell pellets were resuspended with 1 ml fresh-made N-hydroxysuccinimido-biotin in PBS at 50 $\mu\text{g/ml}$. After incubation for 15 minutes at room temperature, the reaction was terminated by washing with 50 ml cold PBS. Cell pellets were resuspended and incubated for 30 min at 4°C in 500 μl of cell lysis buffer containing 50 mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 $\mu\text{g/ml}$ aprotinin, 1 mM PMSF, and 10 mM iodoacetamide. The insoluble debris including nuclei was removed by centrifugation at 13,000 rpm for 10 minutes.

2.16. IMMUNOADSORPTION.

Aliquots of 10^7 biotinylated cells were lysed by addition of 500 μl cell lysis buffer. Aliquots of 500 μl of cell lysate were precleared by incubation with Sepharose 4B beads on the nutator at 4°C overnight. Following centrifugation, the supernatant was collected. The precleared lysate was incubated with shaking at 4°C overnight together with 20 μl of anti-CD45 MI/89.18.7.HK mAb-coupled-Sepharose 4B, which was prepared by conjugating 4 mg mAb to 1 ml swollen CNBr-activated Sepharose 4B. The

precipitate was washed three times with 1 ml cell lysis buffer each time.

2.17. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND THE DETECTION OF CD45 USING BIOTIN-LABELED CELLS.

The immune complexes in the immunoadsorptions were separated from the beads by the addition of 60 μ l 1x concentration of reducing SDS sample buffer containing 0.07 M SDS, 0.1 M Tris, 0.1 M dithiothreitol, and 0.17 M glycine. Samples were boiled for 10 min and centrifuged to separate the supernatant which was fractionated on 7.5% SDS-PAGE gel at 120V using a Mini-PROTEIN II Electrophoresis Cell (Bio-Rad). The SDS-PAGE high range MW standards employed contained myosin, β -galactosidase, phosphorylase b, serum albumin and ovalumin with MW of 200Kd, 116Kd, 97Kd, 66Kd and 45Kd respectively. The dye front was run off the bottom of many of the gels in order to better separate the high molecular weight CD45 isoforms. After the fractionation, the separated material was transferred to nitrocellulose in transfer buffer containing 25 mM Tris, 190 mM glycine, 20% methanol and 0.1% SDS using 30 V at 4°C overnight. The membrane was blocked to ensure saturation of the nitrocellulose binding capacity with 2% BSA in Tris buffer solution-Tween 20 containing 0.05 M Tris, 0.15 M NaCl and 0.1% Tween 20 (TBST) for 1 hour at RT. Following one wash with TBST, the membrane was incubated for 20 minutes with avidin-horseradish peroxidase (Bio-Rad, Hercules, CA) diluted 1:9000 in TBST. After three washes, the ECL (Amersham

International plc, Buckinghamshire, England) reagent was used to detect the signal.

2.18. DETECTION OF CD45 ON YAC-WT AND YAC-N1 USING WESTERN BLOT.

Aliquots of 10^8 YAC-WT and YAC-N1 cells were harvested and washed with PBS. The cell lysis buffer without NP-40 was added, and cells were sonicated and centrifuged to separate the membrane portion from the cytosolic portion. The pellet containing the cell membrane was dissolved by addition of cell lysis buffer with NP-40. The protein was fractionated by SDS-PAGE and transferred to nitrocellulose. The membrane was blocked by incubation with 2% BSA in TBST for 1 hr at RT. The membrane was incubated with a 1:500 dilution of rabbit anti-CD45 anti-serum that recognizes the intracellular portion of CD45 (kindly provided by Dr. Ostergaard) at 4°C overnight. After three washes, HRP-conjugated anti-rabbit IgG at a dilution of 1:1000 was added and incubated for 1 hr at RT. Following extensive washing, the ECL detection system was used.

2.19. INHIBITION OF ANTI-CD45 MAB BINDING TO CD45 ON THE CELL SURFACE BY NATURAL IGG.

The saturating binding concentrations of mAbs were pre-determined by using a single antibody-binding assay. Aliquots of 3×10^5 LYNAb⁺ and L5178Y-F9 were washed

once with 0.1% BSA/PBS pH 7.4 buffer. An aliquot of 100 μ l of purified DBA/2 natural IgG at 0.2mg/ml was added to the cell pellets, or PBS was added as control. The purified IgG was adjusted to the same volume as the starting serum and a $\frac{1}{4}$ dilution equivalent to the $\frac{1}{4}$ dilution of DBA/2 serum used in our regular NAb binding assay, had a protein concentration of 0.2 mg/ml. After 90 min incubation at 4°C and one wash, 100 μ l of a sub-saturating binding concentration of biotinylated anti-CD45RA mAb 14.8, 100 μ g/ml, was added and incubated for 15 min at 4°C. Cells were washed twice and reacted with 100 μ l of 1:80 dilution of R- phycoerythrin (R-PE) conjugated streptavidin for 20 min at 4°C. Following one wash, 200 μ l of 1% paraformaldehyde was added to fix these cells. Control mAbs included biotin labeled anti-pan CD45 I3/2 and biotin-labeled anti-CD45RB MB23G2, both of which were used at sub-saturating binding concentrations, 1 μ g and 100 μ g/ml, respectively.

2.20. INHIBITION OF NATURAL ANTIBODY BINDING TO LYNAB⁺ CELLS BY CD45RABC AND CD45RB/RO.

Aliquots of 3×10^5 LYNAb⁺ cells were washed once with a solution of 0.1%BSA/PBS pH 7.4. An aliquot of 50 μ l of purified natural IgG at 0.2 mg/ml plus 50 μ l of CD45RABC at 1.2 μ g/ml or CD45RB/RO at different concentrations, 0.6, 1.2, 1.8, and 2.4 μ g/ml, were added to the cell suspension and incubated for 1hr at 4°C. The IgG used in this assay was the same as in the previous inhibition experiment. In order to keep the total volume of the solution consistent at 100 μ l, volumes of 50 μ l of IgG and 50 μ l of

purified CD45RABC or CD45RB/RO were chosen. The CD45RABC preparation and the mixture of CD45RO and CD45RB (CD45RB/RO) obtained from Dr. Ostergaard were purified from the A20.CY cells and the EL4 cells respectively. The A20.CY has mostly RABC but does contain other CD45 isoforms since other bands appear on SDS-PAGE fractionation. The EL4 contains mostly RO and RB and predominantly RB (personal communication with Dr. H. Ostergaard). After one wash, FITC-anti-mouse IgG was added and incubated with cells for another 20 min at 4°C. Cells were washed once, fixed and assayed by flow cytometry as before. An anti-CD44 monoclonal antibody IM7 was used as a control to assess the effect of any nonspecific interaction on the cell surface between IgG and purified CD45. The working concentration of IM7 was 5 µg/ml at which a sub-saturating binding activity was achieved.

2.21. STATISTICS.

The Student's t test, t-dependent (td) or t-independent (ti) was used to determine the statistical significance for the differences between specific and control monoclonal antibody binding by the same tumors and for the differences between NAb binding by different tumor lines. P values that were greater than 0.05 were not considered to be significant.

3. RESULTS

In this study, we used an NK-resistant T-lymphoma cell line, L5178Y-F9 as the parental cell line. In order to study the correlated changes between NAb binding and cell surface molecules, a variant LYNAb⁺ was selected for higher NAb binding by FACS (Tough *et al.*, 1988). Our hypothesis is that the increased level of NAb binding is associated with increased numbers of NAb binding molecules. Several molecules including CD45RA have been found to be increasingly expressed on LYNAb⁺ in comparison with L5178Y-F9 (Chow, *et al.*, 1999). Using this model, we further studied the relationship between NAb binding levels and the expression of different isoforms of CD45. In addition, we extended this model to *in vivo* animal experiments by *s.c.* inoculation of syngeneic DBA/2 mice with LYNAb⁺ cells. The *in vivo* selected variants were also tested for changes in NAb binding and expression of CD45 isoforms. Furthermore, some approaches were also employed to search for the direct evidence that NAb may bind to CD45. We compared NAb binding on CD45⁻ YAC cells with CD45 transfected YAC cells and YAC-WT cells. We also used NAb to block anti-CD45RA mAb binding to LYNAb⁺ cells. Finally, purified CD45 was used to inhibit NAb binding to LYNAb⁺ cells.

3.1. ANTI-TUMOR IGG AND IGM IN NORMAL SYNGENEIC DBA/2 SERUM.

L5178Y-F9 and LYNAb⁺, the latter selected through FACS for high IgG and IgM

NAb binding, were incubated in a 1/4 dilution of syngeneic DBA/2 whole serum and stained with FITC-anti-mouse IgG diluted 1/10 or FITC-anti-mouse IgM diluted 1/20 to assess the ability of normal IgG and IgM to recognize the cell surface molecules of these two tumor cells. Although statistical significance was not reached due to the inadequate number of experiments, comparison of NAb binding between these two cells suggested a trend, that LYNAb⁺ bound more IgG, 68.1±14, and IgM, 37.3±14.2, than the parental L5178Y-F9 at 41.3±2.6 and 19.0±8.6 respectively (Table I). In terms of the relative binding of IgG and IgM to these two cells, the ratio of IgG/IgM detected for the LYNAb⁺ was similar at 1.9:1 to that for the L5178Y-F9, 2.2:1. This may indicate that IgG and IgM contributed equally to the increased NAb binding on LYNAb⁺ compared with the L5178Y-F9.

3.2. NAB BINDING AND CD45 EXPRESSION ON L5178Y-F9 and LYNAb⁺.

The NAb binding assay has been employed in our laboratory for years. A 1/4 dilution of whole DBA/2 serum was incubated with L5178Y-F9 and LYNAb⁺, and cells were stained with both FITC-anti-mouse IgG at a 1/10 dilution and FITC-anti-mouse IgM at a 1/20 dilution. Accompanying the increased NAb binding by the LYNAb⁺, the uptake of rat IgG_{2b} anti-CD45RA mAb 14.8 was also increased to approximately twice that of the parental cell (Table II), confirming our preliminary observation with the rat IgM anti-CD45 mAb RA3-2C2 (Chow *et al.*, 1999). In addition, the high NAb binding variant bound 50% more CD45RC reactive mAb DNL-1.9 than the parental cell.

Table I. The binding of IgG and IgM in syngeneic DBA/2 serum to L5178Y-F9 variants^a

Mean MCF \pm SE ^b				
L5178Y-F9			LYNAb ⁺	
(n)	IgG	IgM	IgG	IgM
(3)	41.3 \pm 2.6	19.0 \pm 8.6	68.1 \pm 14.0	37.3 \pm 14.2

^a The table shows three independent experiments.

^b Aliquots of 3×10^5 cells were washed, pelleted and incubated with a 1/4 dilution of DBA/2 pooled serum at 4°C for 1 h. Following 1 wash with 0.02% BSA/PBS buffer, the cells were stained with a 1/10 dilution of FITC conjugated anti-mouse IgG or a 1/20 dilution of FITC conjugated anti-mouse IgM. After another wash, cells were fixed with 1% paraformaldehyde and assayed by FACSCan.

By contrast, the binding of CD45RB reactive mAb MB23G2 was significantly reduced to the levels of control mAb binding, suggesting that CD45RB expression on the high NAb binding variant was lost. Both MI/89 and I3/2, also called anti-pan CD45, were against the common portion of all CD45 isoforms and they detected the total amount of CD45, which was decreased in LYNAb⁺ cells. Thus, the relative patterns of CD45 exon expression by the L5178Y-F9 and the high NAb binding LYNAb⁺ variant were CD45^{hi}RA^{lo}RB^{hi}RC^{lo} and CD45^{lo}RA^{hi}RB^{RC}^{hi}, respectively.

Considering that LYNAb⁺ was selected for high NAb binding from the L5178Y-F9 through FACS and exhibited a reduced tumorigenicity compared to L5178Y-F9 (Tough and Chow, 1988), the higher expression of CD45RA and CD45RC and lower expression of total CD45 and CD45RB in LYNAb⁺ might be associated with the lower tumorigenicity. The parallel changes in NAb binding and CD45RA and CD45RC expression in these two cell lines suggested that NAb might bind with CD45RA and RC. Since NAb exhibits polyspecificity, it may also be possible that the changes in NAb binding and CD45RA and RC expression are just coincidental. NAb may bind to molecules other than CD45RA and RC which were increasingly expressed on LYNAb⁺.

Although both MI/89 and I3/2 were antibodies against the common portion of all CD45 isoforms, the apparently different binding capacity on L5178Y-F9 suggests that MI/89 and I3/2 recognize different amino acid epitopes. Their expression levels may be quite different on L5178Y-F9, but very close on LYNAb⁺.

Table II. Binding of NAb and anti-CD45 mAbs to L5178Y-F9 and LYNAb⁺

Expt. # ^a	Antibodies	CD45 Specificity	Mean MCF±SE ^b		P _{td}	P _{ti}
			L5178Y-F9	LYNAb ⁺		
1(24)	NAb		33.7±9.7	67.0±23.2	<0.00001	<0.00001
2(5)	MI/89.18.7.HK 2.43(IgG _{2b})	pan	179.6±26.7 1.4±1.7	48.6±9.1 1.4±2.0	<0.0005	<0.00001
3(4)	I3/2 2.43	pan	99.4±33.7 0.6±0.5	46.6±15.7 1.1±1.7	<0.01	<0.005
4(7)	14.8 2.43	RA	26.3±10.6 3.8±0.6	51.0±19.0 1.9±1.5	<0.005	<0.01
5(3)	MB23G2 Y13-238(IgG _{2a})	RB	70.9±19.3 2.3±2.0	4.9±0.4 4.5±3.6	<0.05	<0.01
6(3)	DNL-1.9 Y13-238	RC	36.8±5.2 1.3±1.2	57.6±7.9 4.4±2.3	<0.05	<0.05

^a The number of assays performed is indicated in parentheses.

^b Aliquots of 3X10⁵ L5178Y-Y9 and LYNAb⁺ were washed. The cell pellets were incubated with a 1/4 dilution of DBA/2 serum or saturating concentrations of mAbs for 1 hr at 4°C and washed twice, followed by incubation with a 1/15 dilution of FITC conjugated anti-mouse IgG plus a 1/25 dilution of FITC conjugated anti-mouse IgM or a 1/64 dilution of FITC-conjugated anti-rat IgG for another 20 min and washed one more time. Cells were fixed with 1% paraformaldehyde

3.3. CHANGES IN BINDING OF ANTI-CD45 MABS AND NAB DURING *IN VIVO* TUMOR PROGRESSION.

The previous studies showed that tumors obtained from the injection site of threshold *s.c.* inocula of L5178Y-F9 in syngeneic mice exhibited decreased NAb binding (Chow, 1984a; Brown *et al.*, 1986). The threshold tumor dose was determined as the cell number that could lead to tumor growth in less than 100% mice. Since decreases in expression of CD45RA and CD45RC were predicted, the LYNAb⁺ variant which expressed higher levels of CD45RA and CD45RC than the parental L5178Y-F9, was chosen to investigate changes in CD45 expression during growth *in vivo*. We predicted that *in vivo* selection of the higher NAb binding LYNAb⁺ could also result in a variant with reduced NAb binding. The variant may also exhibit the similar relationship between NAb binding and CD45RA and RC expression.

In vivo passage of LYNAb⁺ once, yielded X1SC cells which bound reduced NAb and less CD45RA and CD45RC specific mAbs (Table III). NAb, anti-CD45RA and anti-CD45RC mAb binding to X1SC cells were decreased by 55%, 84% and 44% respectively, compared with that of the LYNAb⁺. In contrast, uptake of the anti-pan CD45 mAb I3/2 was dramatically increased, 2.8 fold, following the single passage *in vivo* and no change was observed in the negligible level of specific binding by the anti-CD45RB mAb MB23G2.

After X1SC cells were cultured *in vitro* for one month, they were re-injected into another DBA/2 mouse to obtain the twice passaged X2SC cells. The X2SC exhibited

similar binding patterns, decreased NAb, anti-CD45RA and anti-CD45RC binding amounting to 52%, 60% and 71% respectively and anti-pan CD45 binding increased 1.7 folds compared to LYNAb⁺ cells. In addition, still no anti-CD45RB binding was observed in X2SC cells (Table IV).

Similarly, variants selected three times were obtained by inoculation of the X2SC. The same trend was observed in X3SC cells which showed 60% less NAb binding, 73% less anti-CD45RA and 68% less anti-CD45RC binding and a 3 fold increase in anti-pan CD45 binding (Table V).

All the changes in antibody binding were marked and also statistically significant. Comparing with variants selected once *in vivo*, the X2SC and X3SC cells did not demonstrate further significant reductions in NAb, anti-CD45RA and anti-CD45RC binding or increases in anti-pan CD45 binding. The lack of anti-CD45RB binding was unchanged through the three selections. The failure to detect CD45RB expression was not due to the mAb or the assay because L5178Y-F9 was tested in parallel and demonstrated positive anti-CD45RB binding (data not shown). Furthermore, the parallel alteration in NAb binding and CD45RA, RC expression strongly suggested that NAb may function through CD45RA and RC. The relative NAb binding and expression of CD45 and CD45R determinants by the variants selected *in vivo* were stable for at least 2 months in tissue culture (data not shown). Therefore, in comparison with the high NAb binding parent, the cells selected three times *in vivo* were CD45^{hi}RA^{lo}RB⁻RC^{lo}NAb^{lo}.

Table III. Binding of NAb and anti-CD45R mAbs to LYNAb⁺ and the X1SC variant selected once *in vivo*

Expt. # ^a	Antibodies	CD45 Specificity	Mean MCF±SE ^b		P _{td}	P _{ti}
			LYNAb ⁺	X1SC		
1(5)	NAb		72.8±8.3	32.9±10.7	<0.01	<0.0005
2(4)	I3/2 2.43(IgG _{2b})	pan	38.9±16.5 3.9±4.2	109.5±32.5 0.7±0.6	<0.05	<0.01
3(4)	14.8 2.43	RA	40.0±6.2 4.7±3.9	6.4±5.8 1.8±2.7	<0.001	<0.0005
4(6)	MB23G2 Y13-238(IgG _{2a})	RB	3.9±4.6 1.0±1.4	5.7±6.0 1.5±1.5	NS	NS
5(8)	DNL-1.9 Y13-238	RC	25.7±0.3 3.7±2.2	14.4±7.5 1.9±1.4	NS	<0.05

^a The number of assays performed is indicated in parentheses.

^b Aliquots of 3X10⁵ were assayed as described in the footnote of Table II.

Table IV. Binding of NAb and anti-CD45R mAbs to LYNAb⁺ and the X2SC variant selected twice *in vivo*

Expt # ^a	Antibodies	CD45 Specificities	Mean MCF±SE ^b		P _{td}	P _{ti}
			LYNAb ⁺	X2SC		
1(6)	NAb		102.3±13.9	49.3±15.8	<0.05	<0.005
2(5)	I3/2 2.43(IgG _{2b})	pan	59.7±17.4 0.7±1.6	103.4±13.2 0.3±0.4	<0.05	<0.005
3(4)	14.8 2.43	RA	46.9±10.5 3.0±1.5	19.1±5.9 1.0±1.3	<0.001	<0.0005
4(7)	MB23G2 Y13-238(IgG _{2a})	RB	2.8±2.2 7.7±5.5	1.4±1.1 7.6±6.1	NS	NS
5(6)	DNL-1.9 Y13-238	RC	31.3±19.7 3.5±2.8	9.2±3.0 2.4±3.8	<0.05	<0.05

^aThe number of assays performed is indicated in parentheses.

^b Aliquots of 3X10⁵ cells were assayed as described in the footnote of Table II.

Table V. Binding of NAb and anti-CD45R mAbs to LYNAb⁺ and the X3SC variant selected three times *in vivo*

Expt. # ^a	Antibodies	CD45 Specificities	Mean MCF±SE ^b		P _{td}	P _{ti}
			LYNAb ⁺	X3SC		
1(10)	NAb		84.6±36.1	33.5±8.5	<0.0005	<0.00001
2(9)	I3/2 2.43(IgG _{2b})	pan	21.8±10.0 0.0	65.6±29.2 0.3±0.5	<0.01	<0.005
3(3)	14.8 2.43	RA	41.1±5.4 6.3±2.8	10.9±8.2 6.7±6.5	<0.005	<0.0005
4(7)	MB23G2 Y13-238(IgG _{2a})	RB	1.5±3.9 0.8±1.3	2.0±3.9 0.7±1.2	NS	NS
5(4)	DNL-1.9 Y13-238	RC	25.2±12.0 3.1±2.5	8.0±2.8 2.0±1.5	<0.05	<0.01

^a The number of assays performed is indicated in parentheses.

^b Aliquots of 3X10⁵ cells were assayed as described in the footnote of Table II.

3.4. EFFECT OF NEURAMINIDASE ON CD45 EXPRESSION AND NAB BINDING ON L5178Y-F9 CELLS.

Sialic acid was found to exist on the glycoproteins of almost all tumor cells (Narayanan, 1994). CD45 is a highly glycosylated protein and NAb binding epitopes can be carbohydrates masked by sialic acid (Nahori *et al.*, 1994; Fernsten *et al.*, 1994; Mamoune *et al.*, 1998). Fernsten's study showed that neuraminidase treatment of CD45 to remove the sialic acid enhanced the binding of anti-CD45 autoantibodies from some SLE sera (Fernsten *et al.*, 1994). Thus, it was important to investigate the dependence of the NAb and anti-CD45 mAb binding epitopes on sialic acid on the cell surface. L5178Y-F9 cells were selected for this assay since all isoforms of CD45 were expressed on the cell surface, whereas no CD45RB was observed on LYNAb⁺ cells. Neuraminidase treatment of L5178Y-F9 to remove sialic acid resulted in increases in binding by NAb, anti-pan CD45 I3/2, anti-CD45RA 14.8, and anti-CD45RC DNL-1.9, but eliminated anti-CD45RB MB23G2 binding (Table VI). This suggested that the epitopes recognized by NAb, I3/2, 14.8 and DNL-1.9 were masked, or otherwise altered, by sialic acid, but that binding by MB23G2 was dependent on the presence of sialic acid. Recognition of sialic acid by MB23G2 has been reported (Lazarovits *et al.*, 1996). The present work describes the first observation of the effect of neuraminidase treatment on I3/2, and 14.8 binding to cells. Considering the lack of binding by the anti-CD45RB MB23G2 and the increased binding by the anti-CD45RA to LYNAb⁺, selection for high NAb binding may preferentially select the cells with low sialic acid expression.

Furthermore, the LYNAb⁺ may even express increased asialo-CD45RB which would not be detected by the MB23G2 mAb. However, desialation affects all molecules on the cell surface, some of which may be NAb targets other than CD45. The removal of negative charges on the entire cell surface can be expected to influence many specific and non-specific interactions. In the case of antigen-antibody reactions at the cell surface, changes in antibody binding may occur due to direct removal of sialic acid from an antigenic determinant and/or due to changes in the charge of the microenvironment of the antigen. The reduced negative charge obtained after removal of sialic acid appeared to enhance the non-specific binding of one isotype control mAb, Y13-238.

3.5. CD45 ISOFORMS EXPRESSED ON L5178Y-F9, LYNAB⁺ AND X2SC.

The mRNA splicing of CD45 causes the formation of at least eight different combinations of these four isoforms, RA, RB, RC and RO, and leads to a wide range of MW, from 180MW to 220 MW. Isoform determination by immunoadsorption may allow us to identify the MW of each isoform on a specific cell line. Especially it may also help us to find out the expression of CD45RO which could not be detected by FACS assay due to the lack of anti-CD45RO mAb.

Biotinylation of the cell surface molecules allowed us to examine the isoform profile of the CD45 in terms of their MW on different cells. MW was determined by loading 5 µl of standard marker beside these lanes. This assay revealed some differences

Table VI. The effect of neuraminidase on binding of NAb, and anti-CD45 mAbs to L5178Y-F9

Expt # ^a	Antibodies	CD45 Specificities	Mean MCF±SE ^b		P _{td}	P _{ti}
			HBSS-L5178Y-F9	Neuraminidase-L5178Y-F9		
1(3)	NAb		43.0±16.6	163.6±31.9	<0.05	<0.01
2(4)	I3/2 2.43(IgG _{2b})	Pan	64.9±7.3	91.5±9.5	<0.005	<0.005
			0.03±0.1	1.7±0.4		
3(4)	14.8 2.43	RA	27.7±3.2	37.0±5.3	<0.05	<0.05
			0.2±0.2	1.4±1.1		
4(5)	MB23G2 Y13-238(IgG _{2a})	RB	26.8±8.5	7.2±3.0	<0.01	<0.005
			8.5±5.6	17.7±7.6		
5(3)	DNL-1.9 Y13-238	RC	19.7±7.8	66.8±8.2	<0.0001	<0.005
			2.1±1.9	19.7±3.5		

^a The number of assays performed is indicated in parentheses.

^b Aliquots of 2.5×10^6 L5178Y-F9 cells were washed twice with PBS to remove the protein in the serum and then resuspended in 380 μ l of HBSS/NaOH buffer (made with 3.9 ml HBSS plus 5 μ l 0.5 M NaOH) with 20 μ l neuraminidase (Type V from *Clostridium perfringens*) added at 100 U/ml, or 400 μ l HBSS/NaOH as control. After incubation for 50 minutes in a 37°C water bath with shaking every 10 minutes, the reaction was terminated by the addition of 4 ml wash medium followed by centrifugation. The binding of NAb and mAbs was analyzed by flow cytometry as described in the footnote of Table II.

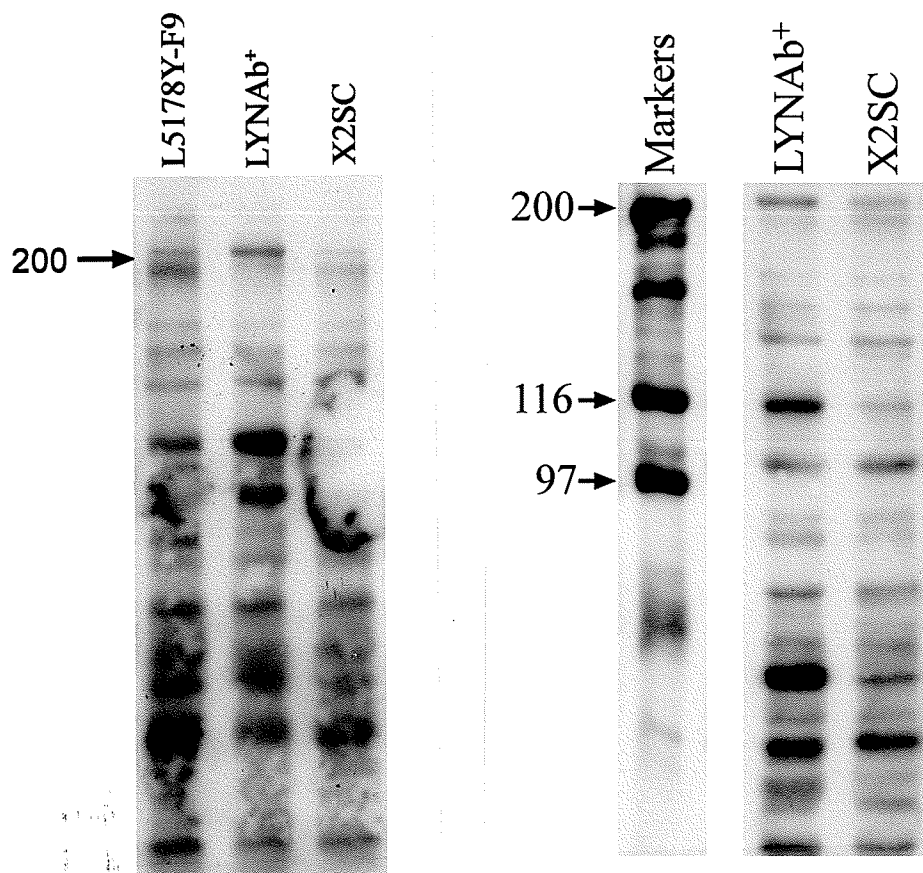


Fig. 1. Detection of CD45 isoforms on L5178Y-F9, LYNAb⁺ and X2SC variant by immunoadsorption.

Aliquots of 10^7 biotinylated L5178Y-F9, LYNAb⁺ and X2SC variant were lysed by addition of 500 μ l cell lysis buffer. CD45 were immunoprecipitated from lysates by addition of 25 μ l anti-pan CD45 mAb MI/89.18.7.HK coupled Sepharose-4B overnight at 4°C, eluted by SDS sample buffer and fractionated on SDS-PAGE. The proteins on the gel were transferred to nitrocellulose and incubated with avidin-HRP followed by ECL detection.

in the range of 180-220 KD where CD45 isoforms were usually located. The L5178Y-F9 and the X2SC expressed predominantly low molecular weight isoforms around 190 KD in addition to some higher molecular weight isoforms around 200 KD isoforms while the LYNAb⁺ expressed predominantly the higher molecular weight isoforms around 200 KD (Figure 1). The use of a molecular weight standard greater than 200 KD would have facilitated the determination of these molecular weights. In addition, bands were detected around 116 KD, 100 KD and 30 KD. The 116 KD and 100 KD bands were likely the α and β chains respectively of glucosidase II which coprecipitates with CD45 and can be surface biotinylated (Arendt and Ostergaard, 1995 and 1997; Baldwin *et al.*, 2000). A 30 KD molecule has also been shown to coprecipitate with CD45 (Arendt and Ostergaard, 1995), and may be a 30 KD adapter protein which has a small external domain. Other lower molecular weight bands may be the result of additional co-isolation or some degradation of molecules during the procedure. Coadsorption with the isotype control revealed no bands in the range of 180-220 KD (data not shown). Comparison of this result with the previous FACS assay suggested that the band around 190KD may represent CD45RB whereas the band around 200 KD may represent CD45RA and RC. Since CD45RO usually exhibits a MW of 180KD, the lack of an apparent band around 180 KD in this assay made the identification of CD45 isoforms more complex. CD45RO may be expressed in none of the cell lines or it may have a higher MW of 190KD due to high glycosylation. In future studies, immunoadsorption with anti-CD45 mAbs against different isoforms could be used to identify the presence of each isoform.

3.6. DIFFERENCES IN THE TUMORIGENICITY AMONG *IN VIVO* SELECTED VARIANTS.

Repeated studies in our laboratory have shown that decreased NAb binding was accompanied with increased tumorigenicity. In addition, a number of human studies revealed that CD45 expression was correlated with tumor prognosis. No animal study has been reported regarding the relationship of tumor progression and different CD45 isoforms. Two assays were performed to determine whether the tumor forming capacity *in vivo* was associated with altered CD45 isoform expression and decreased NAb binding. The previous studies showed that tumor cells derived from threshold s.c. inocula of 50 L5178Y-F9 cells exhibited a significant reduction in sensitivity to NAb and complement (Chow 1984) and s.c. injection of 50 cells of L5178Y-F9 induced tumor growth in 2/8 DBA/2 mice (Sandstrom and Chow, 1987). Thus, 100 LYNAb⁺ cells were inoculated sc into DBA/2 mice. The same number of the *in vivo* isolated X2SC and X3SC cells was tested in parallel.

Figure 2 and Table VII show the tumor frequency and latency, the latter was defined as the length of the period from cell injection until the appearance of the first tumor. Combining the two assays, the total cumulative tumor frequency was 5/12 and 6/12 mice with tumors for the twice and three times selected lines respectively, clearly higher than the high NAb binding parental line with a frequency of 2/12. More impressively, the mean size of the tumors measured 26 days after tumor inoculation showed that the *in vivo* selected variants were markedly larger than the parental LYNAb⁺.

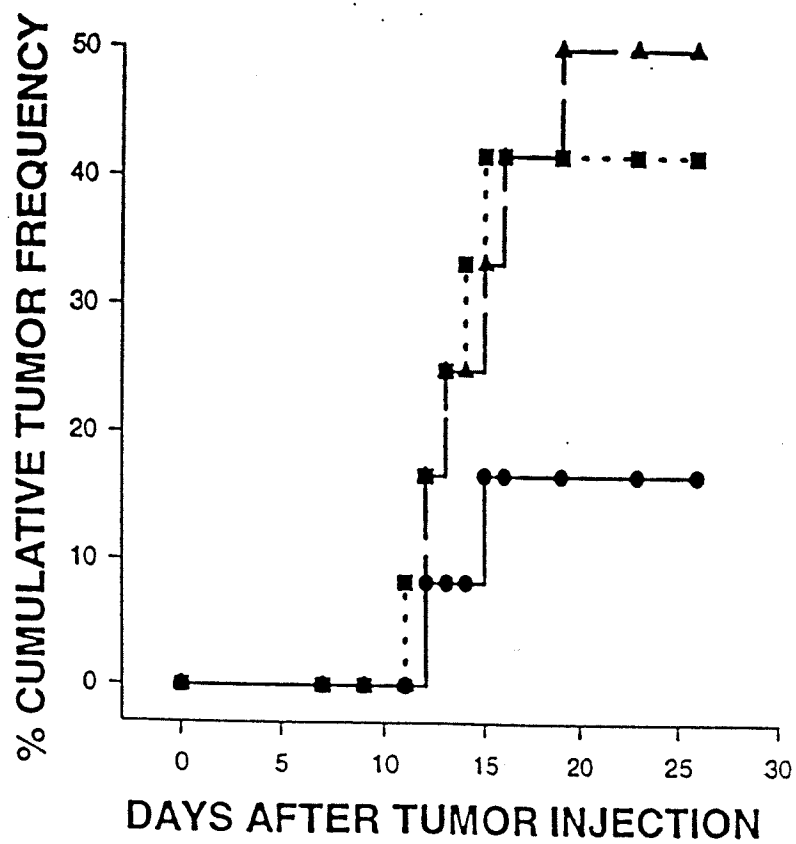


FIGURE 2. Cumulative tumor frequency of in vivo-selected LYNAb⁺ variants. Aliquots of 100 LYNAb⁺ cells (●) and the twice (■) and three times (▲) in vivo-selected variants were injected s.c. into 12 syngeneic DBA/2 mice each.

Table VII. Tumorigenicity of threshold s.c. inocula of LYNAb⁺ and the X2SC and X3SC^a variants selected twice and three times in vivo.

Tumors	No. Mice	No. ^a cells	Days of tumor appearance	mean latency Days ± SE	Accumulative frequency	mean tumor size mm ² ± SE ^b
1 LYNAb	6	100	11	11	1/6	25.0
X2SC	6	100	12	12	1/6	194.9
X3SC	6	100	12,16,19	15.7±3.5	3/6	85.9±25.6
2 LYNAb	6	100	15	15	1/6	28.1
X2SC	6	100	11,13,14,15	13.3±1.7	4/6	140.7±64.1
X3SC	6	100	12,13,15	13.3±1.5	3/6	142.2±124.7
1+2						
LYNAb	12		11,15	13±0	2/12	26.6±2.6
X2SC	12		11,12,13,14,15	13±2.0	5/12	151.1±60.6
X3SC	12		12,12,13,15,19	14.5±2.7	6/12	114.0±86.2

^a An aliquot of 0.1 ml containing 100 cells was injected s.c. into a shaved area in the middle of the lower back for each mouse.

^b Tumor size was assessed as the multiple of the longest diameter and the diameter at 90° measured by calipers 26 days after tumor inoculation.

Thus the CD45^{hi}RA^{lo}RB^{RC}loNAb^{lo} phenotype was linked with increased tumorigenicity.

The variants selected *in vivo* once 1XSC, twice 2XSC and three times 3XSC exhibited about the same reduction in NAb binding, 45.2%, 48.2% and 39.6% respectively. Since we expected that a single selection *in vivo* might not yield cells that would exhibit an increased tumorigenicity which would persist during extended tissue culture, we only inoculated LYNAb⁺ cells into two mice to obtain the 1XSC. Thus we did not have adequate data for X1SC to compare the tumorigenicity with 2XSC and 3XSC. Interestingly, combining the data from these two SC tumorigenicity assays 2XSC and 3XSC, the total cumulative tumor frequency was very close, 5/12 and 6/12 mice respectively. In addition, the mean tumor size of X2SC and X3SC was also similar, 151.1±60.6 and 114.0±86.2, respectively. A single *in vivo* passage cycle enhanced considerably the tumorigenicity of PyV transformed 3T3 cells as compared to their parental cells maintained in the tissue culture (Halachemi and Witz, 1989; Langer *et al.*, 1992). Thus, it was possible that the initial *in vivo* selection might have increased the tumorigenicity.

3.7. NAB BINDING AND CD45 EXPRESSION ON YAC CELL LINES.

The correlated changes between NAb binding and CD45RA and RC expression may suggest that NAb binds to CD45RA and RC. We extended our study to CD45⁻ cells and CD45 transfected cells which may exhibit different levels of NAb binding. We used YAC cell lines including CD45⁺ WT, CD45RABC transfected YAC (YAC-33) and

CD45⁻ variant (YAC-N1) which was obtained through sorting by flow cytometry (Volarevic *et al.*, 1992). The negative expression of CD45 on YAC-N1 was also evident in Western Blot (Fig 3). Tables VIII and IX show that NAb binding to YAC-WT and YAC-33 was 69% and 37% more than to YAC-N1 cells. Therefore, this result also suggests that NAb may bind to CD45.

In addition, the difference in NAb binding between the YAC-WT and YAC-33 versus YAC-N1 was only observed when cells were maintained in a certain concentration range $1-5 \times 10^5$ cells/ml. To get this concentration, cells were usually set at $0.7-1.0 \times 10^5$ cells/ml and cultured for 16-18 hr. The effect of tissue culture on the glycosylation of CD45 has been reported (Ohta *et al.*, 1994).

3.8. CD45 ISOFORMS EXPRESSED ON YAC CELL LINES.

The same method as in section 3.5 was used to identify CD45 isoform expression on YAC cell lines. Similarly to figure 1, figure 4 also shows significant number of bands with lower MW than 116 KD. Because CD45 has so many associated molecules, most of these bands may represent them. In terms of CD45 expression, biotin labeled YAC-WT cells appear to exhibit three different MW isoforms of CD45, 200KD, 190KD and predominately 180KD. Except for the less amounts of CD45 molecules on YAC-33 cells, YAC-33 exhibited a similar isoform pattern as YAC-WT, mainly expression of low MW isoform of 180Kd. However, there is marked discrepancy between this immunoadsorption (Fig. 4) and the Western blot (Fig. 3) in the previous assay. There

were no 180KD and 200KD bands in the Western blot result. The anti-CD45 serum recognizes the intracellular portion of CD45 and it is supposed to react with all the isoforms of CD45. However, it may be possible that the external structure of both low and high MW isoforms of CD45 blocked the reaction between anti-CD45 serum and their intracellular portions.

3.9. NATURAL IGG INHIBITED ANTI-CD45RA MAB BINDING TO THE CELL SURFACE.

Based on the flow cytometry procedure, we made use of cells as an immobilized matrix to stabilize NAb binding. The binding of NAb to their ligands on the cell surface would inhibit the candidate mAbs from recognizing their known target molecules. In order to rule out the non-antibody molecules such as lectins in the serum combining with CD45, it was necessary to utilize purified natural immunoglobulin, such as IgG that was the main component in the normal DBA/2 serum. CD45RA isoform was our target molecule. Although both LYNAb⁺ cells and L5178Y-F9 cells expressed CD45RA (Table II), it was not reasonable to use the low CD45RA expressing L5178Y-F9 cells since greater variation may easily be created at low levels of binding. Thus the LYNAb⁺ cell was used as the target cell.

In this assay, 100 µl of a ¼ dilution of purified natural IgG, at about 0.2mg/ml was incubated with LYNAb⁺ cells for 90 min at 4°C to stabilize the immune complex.

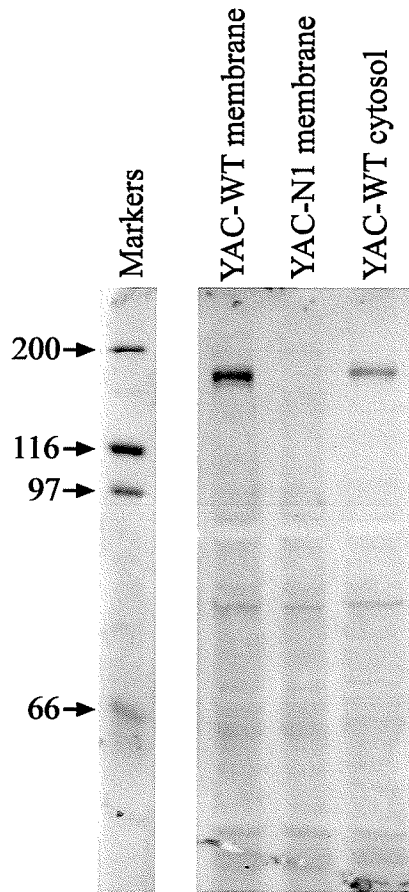


Fig. 3. Detection of CD45 isoforms on the YAC-WT and YAC-N1 by Western Blot.

Aliquots of 10^8 YAC-WT and YAC-N1 cells were harvested and washed with PBS. Cell lysis buffer without NP-40 was added and cells were sonicated and spun down to separate the membrane portion from the cytosolic portion. The pellet containing cell membrane was dissolved by addition of cell lysis buffer with NP-40. The protein was fractionated by SDS-PAGE and transferred to nitrocellulose. The membrane was incubated with 1:500 dilution of rabbit anti-CD45 serum, which recognizes the intracellular portion of CD45, at 4°C overnight. After three washes, HRP-conjugated anti-rabbit IgG at a dilution of 1:1000 was added and incubated for 1 hr at RT. Following extensive washing, the ECL detection system was used to visualize CD45 isoforms.

Table VIII. NAb and anti-pan CD45 binding to CD45⁻ and wild-type YAC cells

Expt # ^a	Antibodies	MEAN MCF±SE ^b		P	Percentage of increas (%)
		YAC-N1/CD45 ⁻	YAC-WT		
1(4)	Nab	50.7±12.9	83.2±19.9	P _{td} <0.005 P _{ti} <0.05	69.4±49.7
2(5)	anti-CD45(I3/2) isotype C(2.43)	1.0±0.6 0.04±0.04	198.5±39.2 0.42±0.35		

^a The number of assays performed is indicated in parentheses.

^b Aliquots of 3X10⁵ YAC-WT and CD45⁻ cells were washed. The cell pellets were incubated with a 1/4 dilution of A/J mice serum or a saturating concentration of I3/2 for 1 hr at 4°C and washed twice, the cells were stained with a 1/10 dilution of FITC conjugated anti-mouse IgG plus a 1/20 dilution of FITC conjugated anti-mouse IgM or a 1/60 dilution of FITC-conjugated anti-rat IgG for another 20 min and washed one more time. Cells were fixed with 1% paraformaldehyde.

Table IX. NAb and anti-pan CD45 binding to CD45⁻ and CD45RABC-transfected CD45⁻YAC cells

Expt. # ^a	Antibodies	Mean MCF \pm SE ^b		P	Percentage of increa (%)
		YAC-N1/CD45 ⁻	YAC-33/CD45 Transfected		
1(6)	Nab	67.6 \pm 12.7	88.9 \pm 2.9	P _{td} <0.05 P _{ti} <0.005	37.1 \pm 36.6
2(4)	Anti-CD45(I3/2) isotype C(2.43)	0.68 \pm 0.24 0.04 \pm 0.04	75.8 \pm 11.6 0.29 \pm 0.23		

^a. The number of assays performed is indicated in parentheses.

^b Aliquots of 3×10^5 YAC-N1/CD45⁻ and YAC-33/CD45 cells were washed. The cell pellets were incubated with a 1/4 dilution of A/J mice serum or a saturating concentration of I3/2 for 1 hr at 4°C and washed twice, the cells were stained with a 1/10 dilution of FITC conjugated anti-mouse IgG plus a 1/20 dilution of FITC conjugated anti-mouse IgM or a 1/60 dilution of FITC-conjugated anti-rat IgG for another 20 min and washed one more time. Cells were fixed with 1% paraformaldehyde.

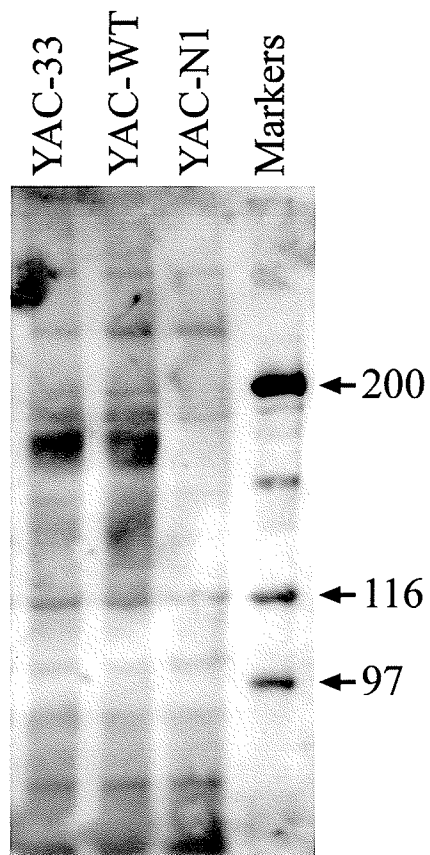


Fig. 4. Detection of CD45 isoforms on YAC-WT, and YAC-33 and YAC-N1 by immunoadsorption.

Aliquots of 10^7 biotinylated YAC-WT, YAC-33 and YAC-N1 were assayed as described in the footnote of Fig 1.

The purified IgG was concentrated to the volume equal to the original serum and a 1/4 dilution of whole serum was used in the NAb binding assay. Firstly a single experiment was done to determine the working concentration of mAbs, at which a reasonably high MCF reading was achieved but at a concentration lower than the saturating concentration. Table X showed the preliminary data of the titration of 14.8, I3/2 and MB23G2. Concentrations of 200, 100, 50 and 25 µg/ml were tested on 14.8, with 200 µg/ml revealing the highest MCF. A working concentration of 100 µg/ml was chosen. Purified DBA/2 IgG preabsorbed with tumor cells was not used as a control because cell surface materials are released during the incubation even at 4°C. Monoclonal antibodies that do not react with the cell surface were also not used as a control in place of purified natural IgG because the natural IgG is an unknown combination of IgG subclasses. Control mAbs were used, I3/2 and MB23G2. I3/2 was examined at concentrations of 20, 10 and 5 µg/ml, with 20 µg/ml exhibiting the highest MCF. Thus, 10 µg/ml was used as the working concentration. I3/2 has the same isotype as 14.8 and binds well to LYNAb⁺. We used L5178Y-F9 as the target cell for MB23G2. The same concentration of MB23G2 was used as for 14.8, 100 µg/ml which may be able to rule out the effect of increased non-specific binding at such a high concentration. In addition, a shorter mAb incubation time of 15 min was considered to be critical to reduce mAb replacement of natural IgG bound to the cells. Table XI showed that natural IgG could inhibit 26.0±3.0% of bio-14.8 binding to CD45RA. The inhibition exhibited statistical significance. In contrast, the inhibitory effects of natural IgG on the binding of bio-I3/2 and bio-MB23G2 were small

at $2.8 \pm 4.7\%$ and $5.8 \pm 4.9\%$, respectively. The differences in the IgG NAb inhibition for the binding of the different mAbs suggests that the inhibition was specific, particularly considering that the pan-CD45 reactive mAb binds the same molecules as the anti-CD45RA and anti-CD45RB but via a different epitope on the molecule. Another independent assay also revealed similar inhibition $29.0 \pm 9.5\%$ of bio-14.8 binding to CD45RA, although no parallel controls of I3/2 and MB23G2 were examined. Therefore this assay may suggest that natural IgG binds to CD45RA.

3.10. PURIFIED CD45 INHIBITED NATURAL IGG BINDING TO LYNAB⁺ CELLS.

Based on the previous inhibition assay, we tried to use purified CD45 to inhibit the binding of NAb to target cells. If natural IgG and anti-CD45RA mAb bind to the same target, purified CD45RA should inhibit the binding of natural IgG. An early ELISA by Chow showed that NAb positively bound to both purified CD45RABC and purified CD45RB/RO. The latter was a mixture of CD45RB and CD45RO. Thus both of these purified CD45 preparations were used in this assay.

The result (Table XII) showed that the binding of 0.2 mg/ml purified natural IgG to LYNAb cells was increasingly inhibited by purified CD45RB/RO at increasing concentrations, from 0.3 and 0.6 to 0.9 $\mu\text{g/ml}$. The optimal inhibition was reached at 0.9 $\mu\text{g/ml}$ and the inhibition at 1.2 $\mu\text{g/ml}$ was same as that at 0.9 $\mu\text{g/ml}$. A mAb IM7 anti-CD44 was used as a control for natural IgG. A single test was done to determine the

saturating concentration of IM7 (Table X). A concentration of 5 $\mu\text{g/ml}$ was chosen as sub-saturating. At the optimal concentration of 0.9 $\mu\text{g/ml}$, CD45RB/RO inhibited IgG binding up to 34%, whereas the control showed zero inhibition. Since a limited amount of CD45RABC was available, we only tested one concentration, 0.6 $\mu\text{g/ml}$, at which CD45RABC inhibited IgG binding up to 39%. However, no control was performed. Though all the inhibition on IgG has reached statistical significance, only one control was done for CD45RB/RO at a concentration of 0.9 $\mu\text{g/ml}$. Therefore, this assay indicated that 0.9 $\mu\text{g/ml}$ of purified CD45RB/RO could inhibit IgG binding to LYNAb⁺ cells. This is consistent with the idea that asialo-CD45RB may be a target of NAb.

Table X. Determination of the working concentrations of biotin-labeled mAbs binding to LYNAb⁺ and L5178Y-F9

mAb	conc. (µg/ml)	MCF	
		LYNAb ⁺	L5178Y-F9
14.8	100	84.5	
	50	56.2	
	25	26.9	
14.8	200	121.2	
	100	99.1	
I3/2	20		102.1
	10		90.2
	5		80.9
IM7	10	75.0	
	5	63.4	
	2.5	63.5	
	1.25	61.0	

Table XI. Natural IgG inhibition of biotinylated anti-CD45 binding to LYNAb⁺ and L5178Y-F9

Exp. # ^a	Cells	CD45 isoform	Conc.	Mean MCF±SE ^b		Inhibition %±SE	P _{td} , P _{ti}
				Specificity of mAb (µg/ml)	PBS		
1. (4)	LYNAb ⁺	14.8-RA	100	54.0±3.4	38.4±3.6	29.0±9.5	0.008, 0.004
2. (4)	LYNAb ⁺	14.8-RA	100	88.8±11.7	65.2±7.41	26.0±3.0	0.0125, 0.0063
(4)	LYNAb ⁺	I3/2-Pan	10	71.5±5.9	69.9±7.2	2.8±2.4	NS
(4)	L5178Y-F9	23G2-RB	100	39.0±4.0	36.9±4.1	5.8±4.9	NS

^a The number of assays performed is indicated in parentheses.

^b 3X10⁵ cells were incubated with 100 µl of 0.2 mg/ml protein A-purified natural IgG or PBS/0.1%BSA for 90 min at 4°C. Cells were washed once and reacted with 100 µl of a sub-saturating concentration of biotinylated anti-CD45 mAbs for another 15 min. Following two washes, cells were stained with strept-avidin-PE for 20 min and fixed with 1% paraformaldehyde.

Table XII. Purified CD45RABC and CD45RB/RO inhibit natural IgG binding to LYNAb⁺

Expt. (n)	Mean MCF±SE ^b			P _{td}	P _{ti}	Inhibition (%)±SE
	IgG	IgG-CD45RB/RO (µg/ml)	IgG-CD45 RABC (µg/ml)			
1 (3)	67.7±4.8	58.8±4.5 (0.3)		<0.02	<0.01	13.1±3.6
		53.8±3.6 (0.6)				20.5±2.9
		49.4±6.1 (0.9)				27.5±4.3
		46.2±6.9 (1.2)				31.9±6.6
2 (4)	68.5±2.1	58.5±1.6 (0.3)		<0.01, <0.01		14.6±10.9
3 (9)	61.5±2.9	41.5±2.3 (0.9)		<0.0001, <0.0001		32.2±2.5
4 (4)	65.9±2.7	45.7±2.9 (1.2)		<0.01, <0.01		30.7±5.9
5 (3)	85.6±12.4		51.2±6.7 (0.6)	NS	<0.04	39.1±6.5
Mean MCF±S.E.						
(n)	IM7	IM7-CD45RBRO (µg/ml)				
(3)	74.6±7.2	80.1±2.8 (0.9)		NS		0

^a Cells were incubated in IgG at 0.1 mg/ml with and without purified CD45RABC or CD45RB/RO, or with IM7 anti-CD44 mAb at 5 µg/ml with or without CD45RB/RO

^b The number of the assays performed is indicated in parenthesis.

4. DISCUSSION

The purpose of the research was to study parallel changes on the NAb binding and the cell surface molecule CD45 expression in association with tumor progression, and to search for the direct evidence that CD45 may be the target of NAb binding. We used the LYNAb⁺ which was selected *in vitro* for high NAb binding. In conclusion, firstly, the selection for high NAb binding was also associated with high CD45RA and RC expression, low pan-CD45 expression and negative CD45RB, suggesting that NAb may bind to CD45RA and RC. Secondly, *in vivo* growth resulted in low NAb binding and CD45RA and RC expression with high tumorigenicity, providing the first animal model showing an inverse correlation between anti-CD45RA, and anti-CD45RC binding with tumorigenicity. Thirdly, changes in NAb and monoclonal antibody binding after neuraminidase treatment of the parental L5178Y-F9 and parallel changes of NAb binding with CD45RA expression in L5178Y-F9, LYNAb⁺ and *in vivo* selected cells suggested that NAb may bind to asialo-CD45RA. Finally, higher NAb binding on YAC-WT and CD45 transfected YAC-33 than on the CD45 negative YAC-N1, inhibition of anti-CD45RA binding by NAb and inhibition of NAb binding by purified CD45RABC indicated that NAb likely bound CD45RA directly.

4.1. SELECTION FOR HIGH NAB BINDING WAS ASSOCIATED WITH HIGH CD45RA AND RC EXPRESSION.

A previous study showed that low NAb binding was associated with high tumorigenicity (Chow *et al.*, 1981). Since our parental L5178Y-F9 cells, an NK-resistant T lymphoma had low NAb binding, it limited our study on the correlation of NAb binding, tumorigenicity and associated cell surface molecules. We selected LYNAb⁺ for high NAb binding by cell sorting after TPA treatment (Tough and Chow, 1988). In addition to increased NAb binding, LYNAb⁺ expressed elevated CD45RA and RC, and decreased pan-CD45. The parallel changes suggested that NAb may bind to CD45RA and RC. It was also possible that selection for high NAb binding may be associated with expression of other cell surface molecule(s) which were accompanied by increased CD45RA and RC expression. In the future, utilization of cell lines transfected with CD45RA and/or RC may provide us additional evidence by comparison of NAb binding on these cells with their parental cells. The IL-2 receptor α chain was also increased on LYNAb⁺ cells (Chow, *et al.*, 1999). Selection for high IL-2R α chain expression with anti-IL-2R α chain mAb from L5178Y-F9 was associated with increased NAb binding as well (Chow *et al.*, 1999). Thus, IL-2R α chain may also bind with NAb. However, CD45RA expression was not elevated. This implies that CD45RA and the IL-2R α chain do not have to be directly associated with each other to bind NAb.

Since anti-pan CD45 mAb binds to the common portion of CD45 molecule, the decreased anti-pan mAb binding and the elevated anti-CD45RA and RC binding in

LYNAb⁺ cells highly suggests that CD45RO isoform may be present and deregulated in LYNAb⁺.

4.2. NAB MAY MEDIATE THE INHIBITION OF TUMOR PROGRESSION.

The criteria of tumorigenicity may include 1) incidence (number of tumor bearers per number of mice inoculated) at certain intervals following tumor inoculation, and 2) the kinetics of tumor appearance which include the average length of the precancer period latency, and the length of the period from cell inoculation to the appearance of the first tumor in the inoculated mouse group (Halachmi *et al.*, 1989).

A previous study by our laboratory showed that tumor variants selected *in vivo* through passage *s.c.*, *i.v.* or *i.p* consistently showed increased tumorigenicity (Chow, 1984). In the present study, the same observation was also found by using LYNAb⁺. LYNAb⁺ cells were injected *s.c.* into syngeneic DBA/2 mice. Tumor cells were recovered and cultured *in vitro* to test their NAb binding. The selection was performed three times and three cell lines were obtained X1SC, X2SC and X3SC. All these *in vivo* selected cells exhibited lower NAb binding and higher tumorigenicity and thus tumor progression in terms of cumulative tumor frequency and mean tumor size. Tough's studies demonstrated that *in vivo* outgrowth of v-H-ras transformed 10T1/2 fibroblasts was associated with less NAb binding capacity and higher tumorigenicity (Tough *et al.*, 1995). The present study confirmed the inverse correlation of NAb binding with tumorigenicity (Chow *et al.*, 1981; Brown *et al.*, 1986; Tough *et al.*, 1995). The

consistent observation indicates that NAb binding can be a valuable means to measure tumorigenicity. Clinically, it may be useful to predict the relative tumorigenicity of early tumors by testing their binding levels with normal NAb, such as IVIg. Such studies may support the idea that NAb plays a significant role in natural defense system against tumors. Cells with higher NAb binding could not survive during the tumor progression *in vivo* and only cells with lower NAb binding could escape from the immune defense system.

The role of NAb in the natural defense system has been studied for years. Recently, the function of NAb and IVIg against tumors has been widely investigated. Normal individuals possessed significantly higher levels of NAb against the tumor-specific molecule MUC1 compared with patients with lung cancer and ovarian cancer (Richards *et al.*, 1998; Hirasawa *et al.*, 2000). Natural anti-neuroblastoma IgM existed in sera of healthy individuals (Fukuda *et al.*, 1999). *In vitro*, IVIg has been found to bind to colon carcinoma, breast carcinoma and squamous cell carcinoma of the lung (Bar-Dayyan *et al.*, 1999). *In vivo*, it has been demonstrated that administration of IVIG into SCID mice inhibits the growth and metastatic lung foci of human melanoma and SK-28 human melanoma cells (Shoenfeld and Fishman, 1999; Shoenfeld *et al.*, 2001). The mechanisms of NAb action against tumor progression have been less understood. Several mechanisms may contribute to the NAb mediated anti-tumor resistance. NAb may function through cytotoxicity including the activation of perivascular complement, induction of neutrophil granulocyte accumulation (Bolande and Mayer, 1990; David *et al.*, 1996; Ollert *et al.*, 1996; Ollert *et al.*, 1997), and the ADCC response (Bar-Dayyan *et al.*, 1999). Bohn

reported that human natural IgM mAb inhibited the proliferation of the cell line of human colon carcinoma *in vitro* and induced complement-mediated cell lysis (Bohn *et al.*, 1994). NAb can also stimulate the production of IL-12 and enhance NK cell *activity in vitro* (Shoenfeld and Fishman, 1999). NAb against neuroblastoma has been found to mediate apoptosis through a tumor specific antigen NB-260 (David *et al.*, 1999). Prasad's study also showed that IVIg induced apoptosis in human monocytic and lymphoblastoid cell lines by directly binding to Fas (Prasad *et al.*, 1998).

In our study model, the parental L5178Y-F9 cell is an NK resistant T cell lymphoma. Thus, NK cells may not always be involved in the inhibition of tumor progression. Macrophages and CTL may play some roles in the anti-tumor resistance. In terms of NAb-mediated anti-tumor progression, more than one mechanism must be involved. Considering the correlated changes in NAb binding and CD45RA and RC expression in L5178Y-F9, LYNAb⁺ and the *in vivo* selected variants, it is highly suggested that NAb may bind to CD45RA and RC. Additionally, both anti-CD45 mAb and galectin-1, a natural ligand for CD45, were able to induce apoptosis in T and B lymphocytes (Perillo, 1995, Fouillit *et al.*, 2000). Furthermore, CD45 is expressed at high levels by most acute myeloid and lymphoblastic leukemias, and anti-CD45 antibody appears to remain on the cell surface and is not easily removed and released into the blood stream (Sievers, 2000). Thus, ¹³¹I-labeled anti-CD45 antibody has been used to deliver radiation to kill the selected target hematopoietic cells (Matthews *et al.*, 1992 and 1999; Sievers, 2000). ¹³¹I-labeled anti-CD45 antibody, combined with traditional cyclophosphamide regimens, followed by bone marrow transplantation has been

successfully used to treat acute myeloid leukemia. Therefore, it is highly possible that NAb may inhibit early tumor progression through CD45RA and RC.

4.3. CD45 MAY PLAY A ROLE IN TUMOR PROGRESSION.

Interestingly, all three *in vivo* selected cell lines exhibited almost complete reductions in CD45RA and CD45RC, no detectable changes in CD45RB and an increase in the total amount of CD45. Considering the higher tumorigenicity of *in vivo* selected cells, the inverse correlation between CD45RA/RC expression and tumorigenicity may suggest CD45RA and/or RC play a role in the tumor progression.

CD45 is highly glycosylated. Cell sialylation affects NAb binding (Mamoune *et al.*, 1998) and is associated with tumorigenicity (Kato *et al.*, 2001). To understand the effect of sialic acid on cell recognition by anti-CD45 mAbs and NAb, we treated the parental L5178Y-F9 with neuraminidase to remove cell surface sialic acid and we found the binding of NAb, anti-pan CD45, anti-CD45RA and anti-CD45RC were increased and the binding of anti-CD45RB was decreased. This finding suggested that the expression of the epitopes recognized by the former three mAbs were reduced by the presence of sialic acid while the anti-CD45RB determinant depended on sialic acid.

The changes in anti-pan CD45, anti-CD45RA and anti-CD45RC binding after neuraminidase treatment seem to be specific. Together the changes in the binding of all of these anti-CD45 mAbs on LYNAb⁺ cells compared to the L5178Y-F9, including negative anti-CD45RB binding, and increased anti-CD45RA and anti-CD45RC binding,

may just indicate decreased sialic acid expression on LYNAb cells. The decreased LYNAb⁺ binding by the anti-pan CD45 which exhibited at least partial reactivity to asialo molecules would support decreased sialic acid expression on the LYNAb⁺ if the level of the lowest isoform CD45RO was decreased. Thus the selection of high NAb binding may preferentially yield those cells with increased asialo CD45RA, RC and RB isoforms, and reduced CD45RO. The presence of predominate higher MW band, 200KD on LYNAb⁺ cells may suggest that there are indeed increased levels of the expression of higher MW CD45 isoforms expressing CD45RA, CD45RB and CD45RC.

CD45RO usually exhibits MW of 180KD. The absence of 180KD band on L5178Y-F9, LYNAb⁺ and X2SC added difficulties to our explanation of isoform profiles in terms of MW. These cells may not express CD45RO, or express CD45RO with high glycosylation, resulting in higher MW 190KD. In addition, the MW of CD45RA, RC and RB can be either 190KD or 200KD. Therefore, the isoform analysis assay could not provide us definitive information regarding the isoform profiles on L5178Y-F9, LYNAb⁺ and 1XSC. Even though, based on the antibody binding assays with and without neuraminidase treatment, we still can assume that LYNAb⁺ might express more CD45RA, CD45RC and probably CD45RB in the asialo form compared with the L5178Y-F9 which likely exhibited each of the CD45 epitopes tested in more sialylated forms. In terms of *in vivo* selected variants, decreased anti-CD45RA and RC binding may only imply increased sialylation on CD45RA and RC. Increased sialylation on the tumor cell surface has been related to the metastatic potential and invasiveness of the tumor cells (Kato *et al.*, 2001; Tanaka *et al.*, 2001). The lack of binding of mAb MB23G2 may

suggest the loss of CD45RB expression on the *in vivo* selected cells or the lack of sialic acid on CD45RB of LYNAb⁺ cells which could not be restored during the *in vivo* selection.

Clinical studies have increasingly focused on CD45 expression on tumor cells. Some reports revealed that the frequency of CD45 expression was related to the prognosis of childhood ALL (Behm *et al.*, 1992). Patients with a high intensity of CD45 expression had adverse prognostic features, whereas patients without CD45 had the highest possibility of survival (Behm *et al.*, 1992; Bolado-Martinez, 1997; Borowitz *et al.*, 1997) and had a more favorable response to therapy (Caldwell *et al.*, 1987; Behm *et al.*, 1992). Suzuki reported that patients with the acute type of adult T cell leukemia (ATL) expressed CD45RO^{hi} populations and exhibited low survival rates and high progression, whereas the CD45RO^{int} populations related to the protection against disease progression (Suzuki *et al.*, 1996, 1998).

Consistent with these observations, a comparison of the LYNAb⁺ and *in vivo* selected variants suggested a new relationship. Higher malignancy may be associated with high CD45 and low CD45RA and RC expression. Clinically, CD45 isoforms may be useful to test the prognosis of human T cell lymphomas. Our studies provide the first animal model suggesting that in general, the CD45RA^{lo} lymphomas are more progressive and exhibit poorer prognosis than CD45RA^{hi}.

It has been demonstrated that CD45 PTPase can activate Lck Src-family kinase activity by dephosphylating the negative regulatory residue Lck-Tyr505 (Ostergaard, 1989; Ostergaard and Trowbridge, 1990), although CD45 can also inhibit src kinase

activity by dephosphylating a positive residue Lck Tyr 394 (Ashwell and D'Oro, 1999; Baker *et al.*, 2000). In our study, the increased total CD45 expression in the *in vivo* selected variants may indicate the possibility of higher PTPase than LYNAb⁺, which appeared to be associated with increased tumorigenicity. However, the effects of CD45 on Src-family kinase activity may vary depending on the cell type and state of activation and differentiation (Ashwell and D'Oro, 1999). It may be possible that other factors, such as glycosylation, ligands and associated molecules may also contribute to the regulation of the ability of the PTPase domains to associate with potential substrates. It would be worthwhile to compare the PTPase activity of LYNAb⁺ and the *in vivo* selected variants.

Other investigators have identified different molecules which were increasingly expressed during tumor growth *in vivo* and were considered to be markers of tumor progression. They include Fcγ receptor RIIBI and IL-1 receptor on polyoma-virus-transformed 3T3 cells (Zusman *et al.*, 1996; Arons *et al.*, 1998), gelatinase and keratin-18 on a clonal cell line of human lung adenocarcinoma (Chu *et al.*, 1997), and MUC18 adhesion molecule on human cutaneous melanoma (Bani *et al.*, 1996). The increased tumorigenicity is also accompanied with lost responsiveness to several growth inhibitors, such as IL-4, IL-6, transforming growth factor-β, IL-1α and tumor necrosis factor-α (Bani *et al.*, 1996). Our study revealed that tumorigenicity was inversely correlated to NAb binding and the expression of CD45RA and RC.

4.4. THE EVIDENCE OF NAB BINDING TO CD45.

The consistent relationship in the changes NAb binding and CD45RA, RC, RB and total CD45 expression in parental L5178Y-F9, LYNAb⁺ and *in vivo* selected variants, in addition to the effect of their sialylation status, provides indirect evidence that NAb binds to asialo-CD45RA and possible asialylated CD45RB as well. A previous ELISA study in our laboratory demonstrated that NAb eluted from tumor cells incubated in normal serum, bound to purified CD45RABC and less to CD45RB/RO (Chow unpublished data). This encouraged us to find direct evidence of NAb binding to CD45.

Firstly, we compared the NAb binding and CD45 expression on CD45 negative YAC-N1 cells, YAC-33 a CD45 transfected YAC-N1 and wild-type YAC-WT cells. Similar to our selection for the higher NAb binding variant from L5178Y-F9, YAC-N1 cells were obtained by negative selection of CD45 from YAC-WT. NAb binding to the YAC-WT and YAC-33 was 69% and 37% higher respectively than that to YAC-N1. Therefore, the higher NAb binding of YAC-WT and YAC-33 than YAC-N1 suggests that NAb binds with CD45 in the context of the cell surface.

Secondly, we used an inhibition assay, which showed that purified natural IgG inhibited anti-CD45RA 14.8 binding to CD45RA by 26%. However, natural IgG only exhibited a very low level of inhibition of the binding of anti-pan CD45 I3/2 and anti-CD45RB 23G2, 2.8% and 5.8%, respectively. Since NAb were characterized by mainly low affinity binding with numerous cell surface molecules (Hurez *et al.*, 1994; Robey *et al.*, 2000), some common approaches including immunoprecipitation to find antibody-

binding molecules were not available to identify NAb binding molecules. Although the flow cytometry analysis showed that NAb bound to molecules on the cell surface, the treatment to separate immune complexes from cell membranes by lysing cells may break up the interaction between antigens and NAb. In this study, we used the cell as a matrix of multiple epitopes and we examined the inhibition of anti-CD45 binding by NAb on the cell surface. This method is based on our routine stable assay for NAb binding. In order to rule out the effect of non-antibody factors such as lectin in the serum, we used purified IgG. By this means, we could keep the natural IgG with higher binding capacity on the cell surface, probably by cross-linking, and we found that IgG partially blocked anti-CD45RA mAb binding to the cell. Considering the possibility that the relatively weak NAb binding may be replaced by higher affinity mAb binding, we used a shorter incubation time with anti-CD45RA. Because the replacement would happen continuously, the actual inhibition may be higher than what we could measure. It may be possible that NAb binds to cells by the Fc portion and the Fab part may bind to anti-CD45 mAb. If this is true, the actual inhibition may even be higher than that shown by our data. More importantly, this method confirms that NAb binds CD45 on the cell surface. Vassilev *et al* (1993) used a similar method to test IVIg binding to CD5, in which cells were stained with saturating concentrations of FITC-labeled anti-CD5 mAb for 1h in the presence or absence of excess of IVIg. However, they did not consider the possible binding of IVIg to anti-CD5 mAb. In addition, since we only used only one concentration of mAbs in this assay which may not be the optimal condition, further study may be necessary to examine the maximal inhibition.

Thirdly, we used another inhibition assay which was also based on our regular NAb binding assay. Mixing purified CD45RB/RO with natural IgG competitively inhibited the natural IgG binding to LYNAb⁺. The inhibition was also specific because CD45RB/RO did not interfere with the binding of an anti-CD44 mAb to cells. This observation may also support the idea that NAb may bind the asialo-form of CD45RB. Recent data by Dr. Chow revealed that human NAb may not bind with CD45RO (Chow *et al.*, 2001). Selection of human Jurket T leukemia cells for high human serum IgG plus IgM binding generated variants which bound not only more anti-CD45RA but also more anti-CD45RB and anti-pan CD45, without changing anti-CD45RO binding (Chow *et al.*, 2001). In addition, the preliminary data showed that purified CD45RABC inhibited purified natural IgG binding to the LYNAb⁺ to some extent. Although limited experiments were done due to inadequate material available, it was really encouraging that some statistical significance was showed at P_{ii} (<0.04). Therefore, NAb may only bind to high MW CD45 isoforms such as CD45RA, CD45RC and CD45RB.

Since the three isoforms, RA, RB and RC that may correlate with NAb binding are represented in the purified CD45RABC preparation, more experiments are necessary. Furthermore, utilization of purified individual CD45RA, RB and RC isoforms in the inhibition assay may finally answer the question whether or not NAb can bind to CD45 and to which isoforms.

The present study is the first report that normal serum contains natural anti-CD45 antibodies which may bind cell surface CD45. The finding is also supported by others' studies (Glotz and Zanetti, 1989; Mimura *et al.*, 1990; Fernsten, 1994). Glotz has

reported a murine hybridoma producing a monoclonal natural autoantibody, 21G10, by fusing neonatal un-stimulated BALB/c splenocytes and the myeloma cell line SP2/0. This autoantibody specifically recognizes the extracellular portion of CD45. Functionally, 21G10 significantly inhibited the ConA-mediated proliferative response of splenocytes (Glutz and Zanetti, 1989). Studies of Mimura and Fersten demonstrate that nearly 1/3 of the serum from patients with SLE contain IgM antilymphocyte autoantibody which specifically binds with the cell surface portion of higher molecular weight isoforms (200KD) of CD45 (Mimura *et al.*, 1990; Fernsten, 1994). The most recent study by Mamoune showed that 25% of sera from SLE patients contained both IgG and IgM, which reacted with the asialylated sugar portion of purified CD45 (Mamoune *et al.*, 1998, 2000).

4.5. NAB MAY REGULATE T CELL ACTIVATION.

TPA treatment of L5178Y-F9 was originally used in our lab to enhance the cell heterogeneity detected in our FACS assay as a variety of NAb binding among individual cells in the whole population. The increased cell heterogeneity allowed us to select the high NAb binding subpopulation. It also activated this T cell lymphoma (Klein *et al.*, 1992). In normal T cells, activation can convert CD45RA⁺ or hi CD45RO⁻ or lo naïve cells into CD45RO⁺ or hi CD45RA⁻ or lo memory cells, but CD45RA expression is rapidly and transiently increased in the early stage (24h) of activation (Yamada *et al.*, 1990, 1992;

Deans *et al.*, 1989). The recent studies showed that a subpopulation CD45RO⁺ or hi cells re-expressed CD45RA⁺ and functioned as long-lived memory T cells (Thiel *et al.*, 1997; Arlettaz *et al.*, 1999). Although the expression of CD45 isoforms on T lymphomas may be more complex than normal T cells, repeated TPA activation of L5178Y-F9 may have provided us with a greater possibility of isolating those highly activated CD45RA⁺ or hi T cells. Furthermore, LYNAb⁺ cells also exhibited increased expression of the IL-2R α chain, an indicator of activated T cells (Tough and Chow, 1988 and Chow *et al.*, 1999). Thus, LYNAb⁺ may be able to represent T cells at a transient stage of activation or long-lived memory T cells. Together with the correlation of increased NAb binding and increased CD45RA, RC expression, it was consistent with the result that NAb may bind to CD45RA and RC. In addition, increased NAb binding was also found for proliferating subconfluent murine C3H 10T1/2 fibroblasts which expressed more PKC in their membrane fractions than resting confluent cells (Wang and Chow, 1999). PKC translocation to the membrane is another marker for activated cells. Tough *et al* (1995) showed that v-H-ras transformed 10T1/2 fibroblasts, which can also be considered as activated cells, exhibited increased NAb binding. Thus, it is highly likely that NAb acts on early-activated T cells.

What may be the outcome of NAb binding to CD45 on normal cells, particularly T and B cells? Most studies have shown that anti-CD45 mAbs inhibit the activation of T cells. *In vivo* administration of anti-CD45 mAb inhibited the humoral immune response by suppressing an early stage of the proliferative response of B cells (Domiasi-Saad *et al.*, 1993). Cross-linking of CD45 with CD2, CD3 and CD28 also inhibited T cell proliferative

responses (Ledbetter *et al.*, 1988). Anti-CD45 mAbs has also been found to inhibit T cell proliferation, IL-2R expression, allogeneic MLR, and the generation and function of CTL (Lazarovits *et al.*, 1992; LeFrancois and Bevan, 1985). By using two fibroblastic cell lines transfected by PKC- β 1 or v-H-ras, Wang and Chow revealed that NAb binding reduced phosphorylation of src (Wang and Chow, 2000). Studies using IVIgG showed that normal homologous and autologous human IgG dramatically inhibited the proliferative response of human PBMC to PHA-activated autologous T cells (Wolf-Levin *et al.*, 1993; Toungour *et al.*, 1996). Therefore, NAb may inhibit the proliferation of some CD45RA^{hi} "super" activated T cells and prevent them going into CD45RO^{hi} stage through binding to CD45RA. Johnnisson also reported that T cells exhibited the highest rate of proliferation at 24 and 48 hr after activation (Johnnisson and Festin, 1995).

In addition, NAb has been found to inhibit the proliferation of other cell lines. *In vitro* study showed that IgA from colostrum down-regulated the proliferation of MCF-10A mammary cells and IVIg inhibited the proliferation of human Jurket cells, J4.1, and Jlg 3.1 (Li and Chow unpublished data). Wang and Chow reported that purified NAb arrested the growth of v-H-ras-transformed 10T1/2, I3T2.1 (Wang and Chow, 2000).

Some studies have revealed that CD45RO⁺ activated T cells were abnormally distributed in various autoimmune diseases. CD4⁺CD45RO⁺ T cells were predominantly present in the peripheral blood and synovial fluid from patients with RA (Mamoune *et al.*, 2000a). They were also found in increased amounts in the peripheral blood and CSF in patients with MS (Barreau *et al.*, 2000). In addition, autoimmune T-cell responses were primarily detected in activated T-cells (CD45RA⁺RO⁺) in newly diagnosed Type I

diabetic patients and in memory T cells (CD45RO) in patients with longer disease duration (Peterson *et al.*, 1999). Therefore, NAb may play a critical role in the control of T cells in normal proliferation and in autoimmune diseases. In terms of the mechanism, NAb may induce the killing of CD45RA^{hi} "super" proliferative T cells by apoptosis, to some extent. The study using auto-anti-CD45 antibodies shows that they can neutralize the activated T cells by anergy or apoptosis (Mamoune, 2000b).

4.6. THE CORRELATION OF T CELL ACTIVATION, AUTOIMMUNE DISORDERS AND THE FORMATION OF TUMORS IN TERMS OF CD45RA AND RO EXPRESSION.

Although activated T cells express increased CD45RA at the early stage of proliferation, both T and B cells will develop into CD45RO^{hi} stage after activation. Interestingly enough, CD45RO⁺ activated T cells were increased and abnormally distributed in various autoimmune diseases. Increased numbers of CD45RO T cells have been detected in synovial fluid from patients with RA (Mamoune *et al.*, 2000a) and in the peripheral blood and cerebrospinal fluid (CSF) in patients with multiple sclerosis (MS) (Barreau *et al.*, 2000) and those with longer disease duration (Peterson *et al.*, 1999). CD45RO⁺ T cells have also been found to predominantly exist in the thyroid tissue of patients with Graves' hyperthyroidism, the lamina propria of Crohn's disease and the joints of reactive arthritis, etc (Mamoune *et al.*, 2000a). CD45RO⁺ T cells exhibited higher adherent ability to endothelial cells than naïve T cells, enhancing trans-endothelial

migration (Mamoune *et al.*, 2000a).

In addition, some highly tumorigenic cells expressed high frequency of CD45RO expression as mentioned above. Is there some relationship between activated cells and autoimmune diseases or even neoplasms? Jensen's study suggested that malignant B cells in Waldenström's Macroglobulinemia and multiple myeloma displayed the phenotype of terminal differentiation, increased CD45RO and decreased CD45RA expression (Jensen *et al.*, 1991a, 1991b, and 1992). Gloghini's study showed that AIDS-related B-NHL exhibited a high frequency of CD45RO expression, suggesting that CD45RO⁺ cells possessed potential autoreactive activity and were preferentially expanded (Gloghini *et al.*, 1995). Taken together, the proliferation of the CD45RO populations may be associated with progression of autoimmune disorders and tumors. Taking into consideration that NAb may bind CD45RA^{hi} T cells which exist in the early stage of T cell activation which may exhibit hyper-proliferation, NAb may play an important role to prevent highly activated cells from changing into the CD45RO^{hi} stage.

4.7. SUMMARY

In order to study the correlation of NAb binding, CD45 expression and tumorigenicity, we selected for a high NAb binding variant by cell sorting from the parental L5178Y-F9, an NK resistant T cell lymphoma which expressed low CD45RA and RC, high CD45RB and pan-CD45. The selected LYNAb⁺ exhibited an increase in CD45RA and RC expression, a decrease in pan-CD45 expression and negative CD45RB. LYNAb⁺ cells were injected into syngeneic mice and *in vivo* selected cells were obtained. The *in vivo* selected variants exhibited high tumorigenicity, low NAb binding and CD45RA^{lo}RC^{lo}CD45^{hi}CD45RB⁻, indicating low NAb binding and low CD45RA/RC were associated with increased tumorigenicity. This also suggests that NAb plays a role in anti-tumor mechanisms. The study also provided a model for staging leukemic disease based on NAb binding CD45 isoforms. Together with neuraminidase treatment, the parallel changes in NAb binding by these cell lines highly suggest that NAb binds to asialo-CD45RA. Finally, by employing inhibition assays, we provided the first direct evidence that natural IgG from normal mouse serum binds to CD45RA. Considering that T cells express CD45RA^{hi} at an early stage of activation and TPA treatment of L5178Y-F9 likely allowed us to preferentially select activated T lymphoma cells, LYNAb⁺ may represent activated T cells. CD45 is a key molecule in T cell activation and for apoptosis of various tumor cells. NAb has been found to inhibit T cell proliferation and tumor growth. Therefore, the present study strongly suggests that NAb acts in the regulation of T cell activation and anti-tumor defense mechanisms through CD45.

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