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**Characterization of the mechanism of target cell recognition
by natural cytotoxic (NC) effector cells using a cloned cell,
L10A2.J: The role of tumor necrosis factor (TNF) and other
determinants**

Matsui, Neil Masato, Ph.D.

University of Hawaii, 1994

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**CHARACTERIZATION OF THE MECHANISM OF TARGET CELL
RECOGNITION BY NATURAL CYTOTOXIC (NC) EFFECTOR
CELLS USING A CLONED CELL, L10A2.J:
THE ROLE OF TUMOR NECROSIS FACTOR (TNF)
AND OTHER DETERMINANTS.**

**A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF
THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF**

DOCTOR OF PHILOSOPHY

**IN
MICROBIOLOGY**

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ABSTRACT

Natural cytotoxic [NC] activity is a type of natural cell-mediated immunity that can kill transformed target cells *in vitro* and is an important mediator of immune surveillance of tumors *in vivo*. Here we have addressed the role of TNF in binding of NC effectors to target cells.

The majority of work in the elucidation of NC effector mechanisms against transformed targets has been carried out with spleen cell NC effectors, i.e. a heterogeneous population. We characterize a cloned NC effector, L10A2.J. L10A2.J is a B cell lymphoma that efficiently kills NC-sensitive targets in an 18-hour ⁵¹Cr-release assay. NC-resistant targets, including targets selected for resistance *in vitro* to spleen cells (10METO.4.4S.3) or tumor necrosis factor [TNF] (10METO.4.5T.1), and targets selected *in vitro* for tumor formation (L88) are also resistant to NC activity. Likewise, targets selected for resistance *in vitro* to L10A2.J (10ME.sL.6) are resistant to spleen cell NC activity and are tumorigenic in normal mice. Furthermore, L10A2.J has properties of NC activity and not of natural killer cells, cytotoxic T lymphocytes, or antigen-dependent cell-mediated cytotoxicity. Finally, L10A2.J-mediated killing is inhibited by antibodies against TNF- α , a lytic molecule of NC effectors. Several transformed cell lines of B lymphocyte lineage also are characterized as NC effectors. All of these B cell NC effectors require effector cell-to-target cell contact for killing to occur. Although 14 of 18 B cell lines tested have NC activity, elimination of B cells with antibody against immunoglobulin plus complement fails to eliminate splenic NC activity.

Using L10A2.J as a cloned NC effector, an assay was developed to assess binding of NC effectors to targets. L10A2.J efficiently binds to 10ME target cells. L10A2.J.sT, a population of L10A2.J selected for reduced binding, has decreased binding to and killing of target 10ME cells. Binding of NC effectors to targets, is therefore important for

efficient killing. Targets that have been selected by spleen cells (10METO.4.4S.3) or L10A2.J (10ME.sL.6) in vitro, or cells that have been selected for tumor formation in vivo (L88) are bound less efficiently by L10A2.J than parent 10ME. Because 10METO.4.4S.3, 10ME.sL.6, and L88 also form tumors, binding of effectors and targets appears to be important in vivo for the immune surveillance of tumors by NC effectors.

TNF is important as a lytic molecule for NC effectors. Killing by L10A2.J and other B cell effectors are substantially inhibited by antibody against TNF. Furthermore, binding of L10A2.J to 10ME can be partially reduced by antibody against TNF or by recombinant TNF. Inhibition of TNF binding to its receptor can reduce binding, but because the inhibition is not complete, it is likely that TNF is only one of many molecules involved in the binding of NC effectors to targets. In addition, binding of NC effectors to targets can be reduced by antibody that blocks the interaction of LFA-1 and ICAM-1, a common leukocyte adhesion system.

The work described here reports two major findings that further our understanding of NC effector-mediated immune surveillance. First, several B cell lines have NC activity, and can be used as cloned NC effectors. Additionally, binding plays a role in the killing of target cells by effectors.

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Chapter 1. Introduction:

The significance of cancer immunology and tumor surveillance by natural cytotoxic effectors

a. The threat of cancer and progress towards a cure

Cancer is one of the leading causes of mortality in the world. In the United States and other developed countries, it is second only to heart diseases (W.H.O., 1990). For women aged 35-74, cancer is the leading cause of death in the United States. The most recent mortality data (1990) shows that of the 2,148,463 deaths in the United States, 505,322, or 23.5 percent, are directly attributable to cancer (Boring et al, 1993). It is estimated that 1.208 million people will have been newly diagnosed as having cancer and 538,000 people will have died of cancer in 1994 in the United States (Boring et al, 1993). Hawaii alone is projected to have 4,100 new cancer cases and 1,800 cancer deaths in 1994 (Boring et al, 1993).

In 1971, the National Cancer Act was signed by then President Richard M. Nixon, declaring war on cancer and projecting a cancer vaccine by 1976. Between 1975 and 1990, however, there has been a seven percent increase in cancer deaths rather than a decrease, and limited vaccines have only recently been tested (Beardsley, 1994). Discounting lung cancer, which is a "self-inflicted" affliction, there is actually a slight decrease in cancer deaths. Indeed, deaths due to certain types of cancers vary widely. Childhood cancers have greatly declined (Beardsley, 1994). Mortality due to other cancers such as those of the colon, rectum, stomach, gall bladder, uterus, cervix, cranium, bone, and testis has declined since 1975 (Beardsley, 1994). However, other cancers such as non-Hodgkin's lymphoma, multiple myeloma, prostate, brain, kidney, esophagus, and breast have actually increased since 1975 (Beardsley, 1994). In spite of this mixed report of progress against cancer, we have learned much about the nature of cancer since 1975.

The differences in progress of stemming cancer deaths are due to the fact that most treatments are tailored to specific cancers. This, in turn is due to the fact that cancers are heterogeneous, connected primarily by the fact that they all involve the deregulated growth of the tumor cells. Currently, most cancers are treated by surgery (38%), radiation (11%), and chemotherapy (6%), or combinations of these (15%) (Menck et al, 1991). Other types of treatment, especially hormone-based treatments, have only recently begun to be employed.

Recent work on cancer immunology has yielded numerous potential therapies. Yet, most of these are in their infancies. Immunological therapies augment and put to use the body's own self defense systems. Immunopharmacology and immunotherapy are the two broad groups of novel treatments proposed by cancer immunologists.

Immunopharmacological treatments include antibody-based therapies, immunostimulants, and cytokines, and have potential in the treatment of cancers (Gallagher, 1993). Early efforts to use antibodies directed at tumor antigens encountered a problem. Monoclonal antibodies, produced in mice or rats, cannot provide a sufficiently strong response in humans and induce a human anti-mouse immunoglobulin [Ig] response (Goldenberg, 1994). Recent research in anti-cancer antibodies has produced humanized antibodies in which the antigen-recognizing regions are derived from rodent monoclonal antibodies, while the remainder of the antibodies (i.e., the F_C region) are derived from human antibodies. Original efforts in producing humanized antibodies consisted of splicing variable region genes from mouse hybridomas to human Ig γ 1 or γ 4 heavy region genes (Winter and Harris, 1993). Currently, the cloning of mouse hybridoma complementarity determining region DNA onto human antibody genes has shown promise and several antibodies are being tested for clinical use such as those against Lewis-Y, p185^{HER2}, PLAP, and CEA tumor antigens as well as the IL-2 receptors of leukemias and lymphomas (Winter and Harris, 1993). Uses for humanized antibodies

include detection, blocking, and initiation of complement- and cell-mediated killing. Furthermore, antibodies themselves have been modified to kill tumor antigen-bearing cells by cross-linking them to toxins such as diphtheria toxin, *Pseudomonas* exotoxin, ricin, and ribosome inactivating protein (Vitetta et al, 1993). Currently, immunotoxins against lymphomas, leukemias, metastatic melanoma, metastatic breast carcinoma, colorectal carcinoma, and ovarian carcinoma are undergoing clinical trials (Vitetta et al, 1993). Radioimmunoconjugates have also been tested for use in radioimmunodetection and radioimmunotherapy (Goldenberg, 1994). Immunotoxins and radioimmunoconjugates have a major advantage over other forms of cancer treatment in that these methods are targeted to the cancer site by the antibodies. This property alleviates the problem of side effects observed in current prevalent cancer treatments such as chemotherapy and radiotherapy.

Immunostimulants, molecules derived from microbial sources, can stimulate an immune response. Glycans and mycobacterial cell wall components enhance macrophage tumoricidal activity and release of cytokines; lipopolysaccharides likewise stimulate B lymphocyte and macrophage activity; and several other microbial biomolecules stimulate interferon production (Hadden, 1993). Administration of cytokines, such as IL-2 and TNF, does not work very well in cancer treatment (Rosenberg et al, 1986). Due to the pleiotropic nature of these cytokines, doses which are therapeutic for cancer treatment have severe side effects (Rosenberg et al, 1986).

Much interest has been generated recently over immunotherapy. The boosting of the cellular immune system has been approached in several ways. Cancer vaccines using irradiated, modified autologous or allogeneic tumor cells have proven somewhat successful against certain types of cancer (Pardoll, 1993). Use of antigen-specific tumor vaccines have met only limited success (Pardoll, 1993).

Lymphokine-activated killer cells are created by IL-2 stimulation of lymphocytes taken from the peripheral blood of the cancer patient (Rosenberg et al, 1986). Similarly, tumor-infiltrating lymphocytes are IL-2 stimulated lymphocytes derived from tumors of patients (Rosenberg et al, 1986). Adoptive transfer treatments using lymphokine-activated killer cells and tumor-infiltrating lymphocytes have proven to be somewhat effective, but have the disadvantage that they are tailored to treat only a single individual (Rosenberg et al, 1986).

Gene therapy of cancer is a recent advance and appears to be one of the most promising fields that takes advantage of tumor immunology knowledge. One of the major problems with tumor immunology is that tumors are fairly nonimmunogenic. Cytokines have been used in gene transfer experiments to boost the immunogenicity of tumor cells (Colombo and Forni, 1994). Transfer of IL-1, IL-2, IL-4, IL-6, IL-7, IFN- α , IFN- γ , TNF, G-CSF, and MCP genes into tumor cells have demonstrated that stimulation of host immunity is possible with cytokine gene-transfected tumor cells (Colombo and Forni, 1994). The other major use of gene therapy for cancer investigation has been for gene marking. In this procedure a marker gene, such as the neomycin-resistance gene, is transferred into the population of leukocytes (Rosenberg, 1992; Morgan and Anderson, 1993). The population of interest can then be tracked to determine if it will infiltrate tumors (Rosenberg et al, 1990). Gene therapy, like tumor-infiltrating lymphocytes, holds some promise but in its present state can only be used to treat specific tumors.

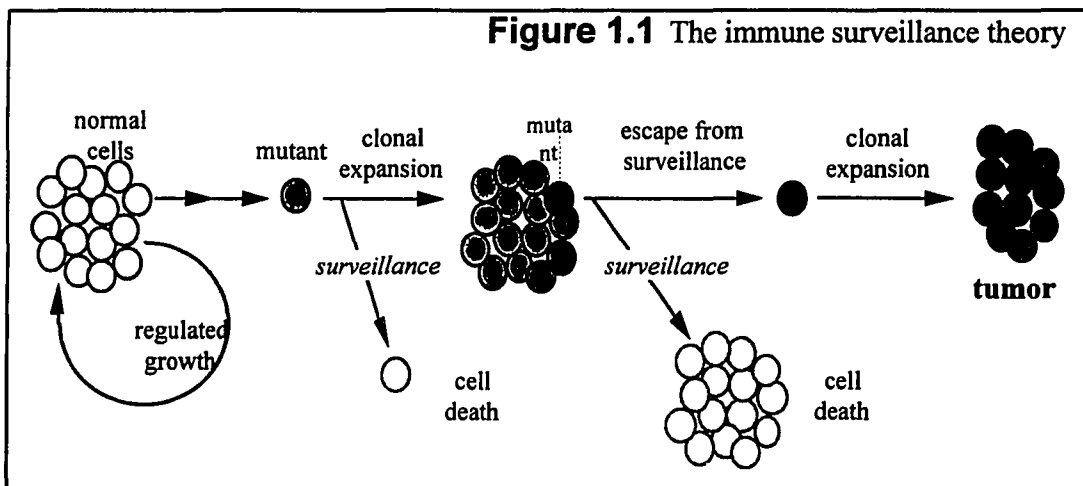
Cancer research has made some progress. However, because of the heterogeneity of tumors there is much to be achieved in the way of broad therapies. In our study we will focus on a relatively new and unstudied field of cancer immunology. Because of the novelty of this field, much of the research is basic. Yet, there is potential for future cancer therapies based on the knowledge gained by basic cancer immunology research.

b. A model for the immune surveillance of tumors

Normal cells are highly regulated. In order for a cell to progress to a tumor, it must overcome internal regulation (gene repair mechanisms and genetic regulation of growth genes) and external regulation (endocrine growth regulatory signals and immune surveillance). The genes and hormones involved in the transformation of cells and progression toward cancer have been extensively studied. Interest in the immunologic regulation, or immune surveillance of tumors, has recently become more important.

The Immune Surveillance theory proposes that, in a normal person, cells often become transformed but, the transformed cells are recognized by the immune system which kills the aberrant cells (Burnet, 1970):

"The concept of immunological surveillance can be stated as follows: In large long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character." (Burnet, 1970).



Under this concept, for clinical cancer to arise, a few of the transformed cells are selected by the immune system such that they are able to avoid surveillance. These variant cells are able to grow, free of interference from the immune system, and expand to form a

tumor (Fig. 1.1). The first step in this model is the transformation of a growth regulatory gene by chemical, physical, or spontaneous mutation. The resulting initiated cell is not immediately tumorigenic. In many cases, cells remain dormant. If conditions are right, the cell may become activated by a promoter. Promotion may be due to a second mutation, hormonal stimulation, chemical stimulation, or physical stimulation. At this stage, the cells will express a transformed phenotype. The transformed cell expands clonally, but is usually limited in its growth. At this point the expanding cell can continue undergoing further mutations. There are also several factors that play a role in selecting cells that are able to grow as tumors. Cells which gain an independence from external regulation will be able to grow as a tumor. One type of external regulation is immune surveillance.

When Burnet proposed his Immune Surveillance Theory, the predominant immune effectors that were known were B cells and T cells and Burnet guessed that T cells would be the major defenses against cancer.

<i>Table 1.1. NCMC Effectors</i>	
◆	natural killer [NK] cells
◆	natural cytotoxic [NC] cells
◆	activated macrophages
◆	lymphokine-activated killer [LAK] cells

However, early evidence indicated that T lymphocytes do not play a major role in normal surveillance (Gillette et al, 1975). Recent work has indicated that an ill-defined class of immune system cells may play a role in normal surveillance of a broad range of precancers. Natural cell-mediated cytotoxic [NCMC] activity has been found in four major classes, NK effectors, NC effectors, activated cytotoxic macrophages, and lymphokine-activated killer cells (Table 1.1). NCMC effectors are natural immune effectors, as opposed to adaptive immune effectors such as B cells and T cells. They are not induced by specific antigen and lack immunological memory (Table 1.2). Furthermore, NCMC effectors lyse certain target cells indicating a limited recognition

repertoire. NCMC activity has been implicated in the surveillance of tumors (Patek et al, 1978; Roder and Haliotis, 1980; Hanna and Burton, 1981; Collins et al, 1981; Patek and Collins , 1988). Both NK and

Table 1.2. Properties of Natural Cell-Mediated Cytotoxic [NCMC] Effectors

- ◆ lyse certain target cells
- ◆ have a very limited antigen recognition repertoire
- ◆ lack immunological memory
- ◆ lack inducibility by antigen

NC activities are composed of a heterogeneous population of cells which makes study of the recognition and lytic mechanisms difficult.

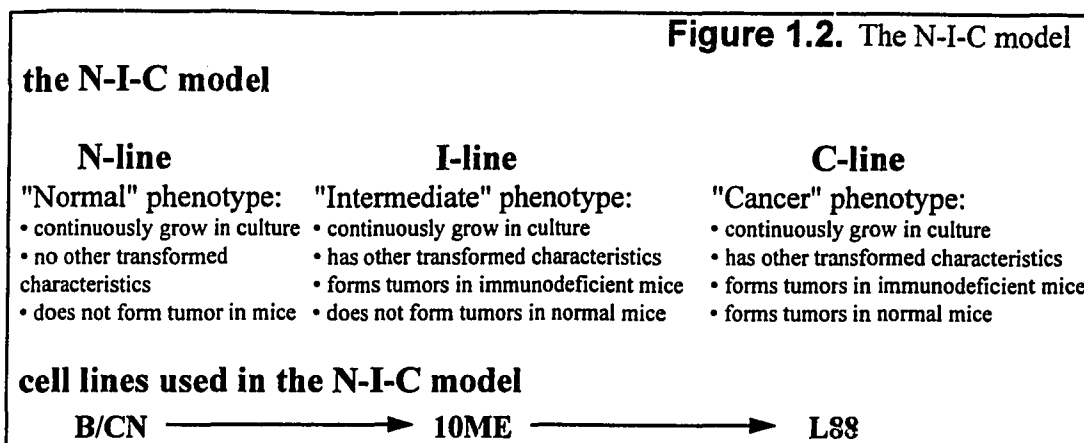
Several differences between NK and NC cells have been determined (Table 1.3). One of the major differences between NK and NC activity is that each kills a particular type of target cell. NK effectors typically lyse transformed cells of lymphoid origin and metastatic cells. It is conceivable that NK activity has evolved as a means to regulate leukocyte-derived transformed cells. NC effectors typically recognize solid, plastic-adherent, transformed cells. Furthermore, NK effectors but not NC effectors can be depleted by antibody against H-2, Thy-1, Lyt-1, NK-1, Qa-4, Qa-5, or asialo GM-1 plus complement (Stutman and Lattime, 1981). NC effectors but not NK effectors can be depleted by anti-NC-1.1 (Smart et al, 1990a; Smart et al, 1990b). Various chemicals and biochemicals have differential effects on NK and NC effectors. Finally, NC activity has a four to seven hour lag in killing with maximum killing at 14-24 hrs, while NK activity is immediately lytic.

NK target recognition (Storkus and Dawson, 1991; Hofer et al, 1992) and lytic mechanisms (Herberman et al, 1986) have been well studied. However, the mechanisms of recognition and cytotoxicity by NC are not well studied.

Table 1.3. Some Differences Between Natural Cytotoxic Activity and Natural Killer Activity

Feature	NC		NK
Mouse strain distribution	different than NK		different than NC
nude, CBA/HN, C3H/HeJ	Yes		Yes
beige, SJL	Yes		Low
Age of appearance in mice	Birth to 4 weeks		4 weeks or more
Age related decline in mice	None		Six months
F _c receptors	No		Yes
Surface antigens	Ab + C'	FACS	Ab + C'
H-2	Low	High	High
Thy-1	No(?)	High	Yes(variable)
Lyt-1	No		Yes
NK-1	No		Yes
Qa-4	No		Yes
Qa-5	No	High	Yes
Asialo GM-1	No		Yes
sIg	No	Some	No
L3T4	No	Some	No
NC-1.1	Yes		No
In vivo effect of:			
⁸⁹ Sr	None		Decreases activity
Estrogen	None		Decreases activity
Cyclophosphamide	None		Decreases activity
Silica	None		Decreases activity
Carrageenan	None		Decreases activity
Glucocorticosteroids	Transient decrease		Transient decrease
In vitro effect of:			
Glucocorticoids	Decreases activity		None
Protein synthesis inhibitors	Increases activity		None
In vitro kinetics of lysis	4-7 hr. lag then linear		linear from t=0

Modified from O. Stutman and E. Lattime, 1981.



c. Tumor surveillance by natural cytotoxic cells

Patek et al have developed a model for analysis of tumorigenesis (Fig. 1.2) (Patek et al, 1978). In this model, normal [N] cells first become transformed by damage to their regulatory genes. The resulting cells are intermediate [I] cells on the pathway to cancer in that they express transformed phenotypes such as anchorage-independent or density-independent growth. These I cells also are able to grow as a tumor in immunodeficient mice but not in normal mice. Furthermore, I cells can be selected by the immune system to become cancer [C] cells. C cells express transformed phenotypes and are able to grow as a tumor in both normal and immunodeficient mice. Experimentally, NC activity has been demonstrated as a means of surveillance using the N-I-C model (Collins et al, 1981). B/CN, the N line, is resistant to NC activity and does not form tumors; 10ME, an I line derived from B/CN, is sensitive to NC activity and forms tumors only in immune deficient mice; and L88, a C line derived from 10ME, is resistant to NC activity and forms tumors in both immune deficient and normal mice.

NC activity, therefore, is an immune surveillance mechanism and is demonstrated in the B/CN-10ME-L88 mouse model. Furthermore, similar NC activity has been described for humans (Collins et al, 1987). Any elucidation of murine NC mechanisms has the potential to bring about novel immunotherapies of human cancer.

d. Tumor necrosis factor

Tumor necrosis factor- α [TNF] is a member of the TNF cytokine family of effectors (Smith et al, 1994; Beutler and Vanhuffel, 1994). It is produced by a number of cell types (Table 1.4), of which the most prominent is the activated macrophage (Urban et al, 1986). In addition, T lymphocytes and B

Table 1.4. Cells which produce TNF

cells	TNF type ^a	ref ^b
activated macrophages	sTNF, mTNF	1-5
T lymphocytes	mTNF, sTNF	1, 3, 6-10
B lymphocytes	sTNF, mTNF	1, 3, 11-14
mast cells	sTNF	3
polymorphonuclear leukocytes	sTNF	3
keratinocytes	sTNF	3
astrocytes	sTNF	3
smooth muscle cells	sTNF	3
intestinal paneth cells	? (express mRNA)	3
tumor cells	sTNF, mTNF	3, 15

^a sTNF= secreted TNF, mTNF= membrane-associated TNF

^b references

1. Dekossodo et al, 1994.	9. Ferreri et al, 1992.
2. Spengler et al, 1993.	10. Santis et al, 1992.
3. Vassalli, 1992.	11. Matsui and Patek, 1994.
4. Ding et al, 1990.	12. Lopez-Cepero et al, 1994.
5. Urban et al, 1986.	13. Goldfeld et al, 1992.
6. Wang et al, 1994.	14. Laskov et al, 1990.
7. Ratner and Clark, 1993.	15. Vanhaesebroeck et al, 1991.
8. Macchia et al, 1993.	

lymphocytes have been found to produce TNF (Laskov et al, 1990; Ferreri et al, 1992; Vassalli, 1992; Dekossodo et al, 1994). Originally described as a factor that causes necrosis of tumors and a factor that causes cachexia, several other systemic effects have been found to involve TNF (Table 1.5) (Carswell et al, 1975; Beutler et al, 1985; Fiers, 1991; Vassalli, 1992). Among the other more widely studied TNF effects is septic shock, endotoxic fever, and resistance to intracellular bacteria and viruses (Tracey et al, 1987; Kawasaki et al, 1989; Fiers, 1991; Vassalli, 1992).

Several forms of TNF have been described (Fig 1.3A). The 17 kDa secreted and 26 kDa integral membrane TNF appear to be transcribed from the same gene (Luettig et al, 1989). The 17 kDa form can also be membrane-associated, although the mechanism

for this association is unclear (Luettig et al, 1989). TNF is normally found as a trimer (Eck and Sprang, 1989). Tumor necrosis factor- β , also referred to as lymphotoxin [LT], is closely related to TNF- α and is likewise found in two forms, LT- α and LT- β (Browning and Ribolini, 1989; Smith et al, 1994; Beutler and Vanhuffel, 1994). LT- α is a 25 kD secreted form and a membrane form is also found as a LT- α /LT- β heterodimer (Crowe et al, 1994). TNF and LT belong to the TNF family of cytokines which include TNF, LT- α , LT- β , Fas ligand, CD40 ligand, CD30 ligand, and CD27 ligand (Beutler and Vanhuffel, 1994; Smith et al,

1994). Both TNF and LT can exert their effects on two TNF receptors [TNFR]: the p55 TNFR, also known as TNFR α , TNFR1, or p60 TNFR; and the p75 TNFR, also known as TNFR β , TNFR2, or p80 TNFR (Tartaglia et al, 1991; Brownell et al, 1992). Both TNFRs belong to the TNFR/NGFR receptor family that includes both TNFRs, NGFR, Fas, CD40, CD30, and CD27 (Beutler and Vanhuffel, 1994; Smith et al, 1994).

Numerous genes and signals have been found to be induced by this highly pleiotropic cytokine (Table 1.6 and 1.7, respectively). TNF-induced genes appear to fall into four categories: 1) cell and matrix damage repair, 2) protection from apoptosis, 3) cell metabolism (which may also play a role in protection from apoptosis, and 4) the

Table 1.5. General effects of TNF

effect / involved in:	ref^a
necrosis of tumors	1, 2-3
cachexia	1, 4
septic shock	1, 5-6
endotoxic fever	1, 7
resistance to bacterial infection	5
resistance to viral infection	5, 8-10
activation of HIV	5, 11
graft-versus-host disease	5, 12
autoimmune reactions	5
anorexia	13
menstruation	14
tumor promotion	15

^a references

- | | |
|---------------------------|-----------------------------------|
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| 2. Creasey et al, 1976 | 11. Qian et al, 1994. |
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Figure 1.3. TNF apoptotic signal transduction.

references:

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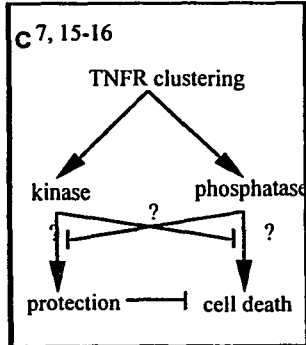
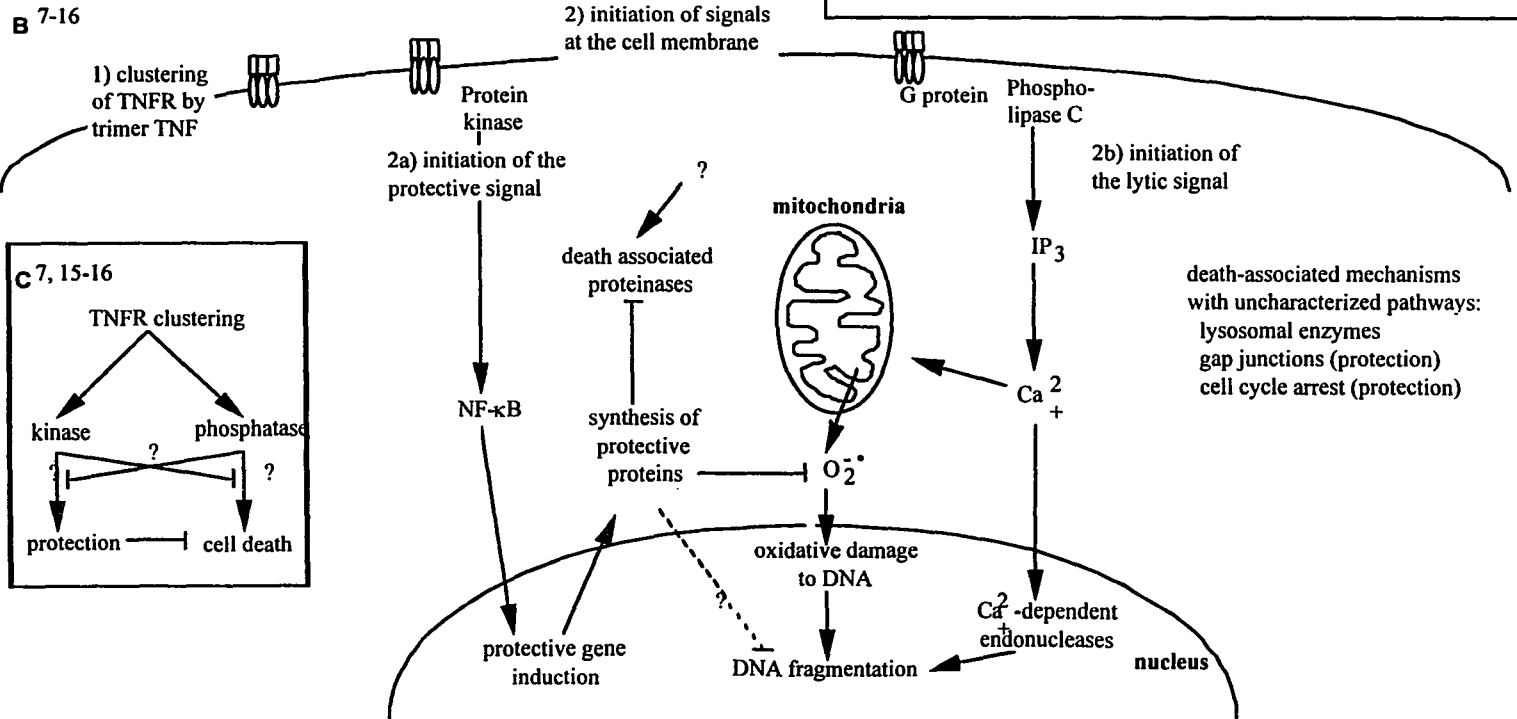
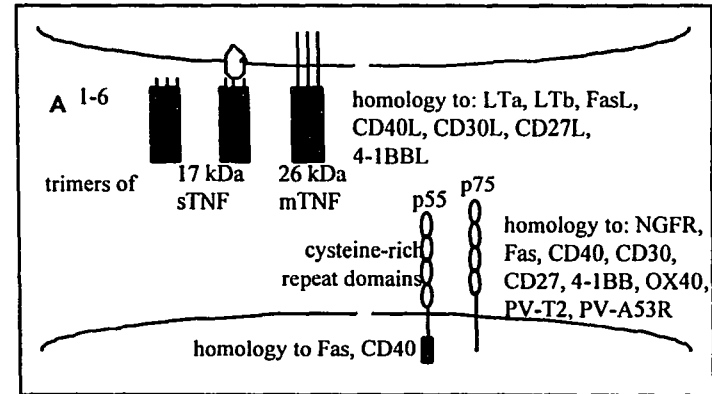


Table 1.6 Known TNF-induced genes

gene	ref ^a	gene	ref	gene	ref
Mn-SOD	1	IRF-1	7	vimentin	8
PAI type-2	2	collagenase	7	collagen tpI	8
A20	3	IL-8	7	<i>rhoB</i>	8
HIV	4	MCAF	7	VCAM-1	8
HLA	5	stromelysin	7	cyto. c ox III	8
glutathione	6	JE	8	NADPH dehyd. I	8
<i>c-fos</i>	7	collagen tpIV	8	ATPase VI	8
<i>c-myc</i>	7	EF-1a	8	other uncharacterized	7, 8

^a references

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3. Opirari et al, 1992.	6. Zimmerman et al, 1989.	

inflammatory response (Lee et al, 1990; Vilcek and Lee, 1991; Gordon et al, 1992). Aside from induced genes, TNF exerts several effects that don't appear to require transcription and translation. TNF causes both apoptotic death and necrotic death (Laster et al, 1988; Woods and Chapes, 1993; Beyaert and Fiers, 1994). Necrotic death involves cell swelling, destruction of organelles, and lysis. Apoptotic death appears to require active participation of the dying cell, and involves cell shrinking and DNA fragmentation. The p55 TNFR, which shares cytoplasmic homology with Fas and CD40, but not with p75 TNFR, appears to be the molecular mediator of the apoptotic signal (Itoh et al, 1991). In addition to cell killing, TNF can cause proliferation, activation, or differentiation of cells. The cellular effect that is observed depends partly on the cell involved and also on other signals. Furthermore, TNF appears to cause shedding of p75 TNFR and internalization without recycling of p55 TNFR (Higuchi and Aggarwal, 1994b).

Apoptotic death of cells induced by TNF has been highly researched, and, again, the mechanisms seem to have a slight degree of variation by cell type. However, an overall view has been determined primarily by using the cell line L929, although most of the events observed in L929 have also been observed in other cell lines (Figure 1.3B)

Table 1.7. Molecular and cellular effects of TNF

effect	pathway	ref
kill transformed cells by apoptosis	p55 only; G protein; protein kinase, countered by a phosphatase or PKC; increased intracellular calcium; superoxide generation in the mitochondria, countered by glutathione; DNA fragmentation; lysosomal enzyme activation	1-15
kill transformed cell by necrosis	?	2, 14
protection from apoptosis	p55; NF- κ B; MnSOD, plasminogen activator type-2, A20 zinc-finger protein (countered by protein synthesis inhibitors)	7, 16-17
kill virus-infected cells	p55	18-20
cell differentiation	p75 and p55; sphingomyelase induced; ceramide-activated Ser/Thr protein kinase (97 kDa); <i>myc</i> repressed	1, 21-23
mitogenesis	p55 or p75; countered by a phosphatase	7, 10, 24
endothelium activation	p55; induce E-selectin, VCAM-1, ICAM-1	25
leukocyte recruitment	?; E-selectin induced	26
class II MHC expression	?; ceramide-activated Ser/Thr protein kinase (97 kDa); TIC-X transcription factor induced	22, 27
HIV activation	?; NF- κ B	28
"ligand passing"	p75 receptor to p55 receptor	29-30
regulation of TNF and TNFR expression	p75	31
internalization of p55	p55	30
shedding of p75	p55	30, 32

- | | | |
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| 2. Woods and Chapes, 1993. | 13. Zimmerman et al, 1989. | 24. Gehr et al, 1992. |
| 3. Beyaert et al, 1993. | 14. Laster et al, 1988. | 25. Slowik et al, 1993. |
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| 5. Wright et al, 1992. | 16. Opipari et al, 1992. | 27. Panek et al, 1992. |
| 6. Watanabe et al, 1992. | 17. Kumar and Baglioni, 1991. | 28. Qian et al, 1994. |
| 7. Tartaglia et al, 1991. | 18. Carpenter et al, 1994. | 29. Tartaglia et al, 1994. |
| 8. Akimaru et al, 1992. | 19. Wong et al, 1992. | 30. Higuchi and Aggarwal, 1994b. |
| 9. Bellomo et al, 1992. | 20. Kenyon et al, 1991. | 31. Higuchi and Aggarwal, 1992. |
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(Beyaert and Fiers, 1994). The initiation of the signal begins with the clustering of TNFR by trimeric TNF. p55 TNFR appears to mediate most major responses of the target cell (Tartaglia et al, 1991; Wong et al, 1992; Higuchi and Aggarwal, 1994a). p75 TNFR may play a role in aiding the binding of TNF to p55 TNFR by a "ligand passing" mechanism (Higuchi and Aggarwal, 1992; Tartaglia et al, 1994).

Once clustering of TNFR occurs, there are two major pathways actuated. The lytic signal is induced possibly via a G protein and phospholipase C. Inositol triphosphate (IP₃) is generated, which causes an increase in the free intracellular calcium ion [Ca²⁺] concentration. Intracellular calcium can affect oxidative metabolism in the mitochondria resulting in the production of superoxide radicals [O₂⁻]. Superoxide radicals can cause general oxidative damage, with the most recognizable being DNA damage. In addition, free intracellular calcium increase is also followed by an increase in nuclear calcium ions. Ca²⁺-dependent endonucleases are activated which also appear to cause DNA damage. Other damage may be mediated by lysosomal enzymes activated via an uncharacterized signaling pathway.

In most TNF-resistant cells, clustering of TNFR by TNF also results in the activation of a protective pathway which appears to be initiated by a protein kinase. The protein kinase activates cleavage of NF- κ B resulting into an activated transcription factor. The transcription factor activates certain TNF-inducible genes including protective genes (Smith et al, 1994). Manganous superoxide dismutase is the most characterized among these. It can detoxify superoxide radicals. Plasminogen activator inhibitor type-2 also protects the cell, probably by interfering with signaling pathways that lead to the production of proteinases. The A20 zinc-finger protein has also been found to protect against apoptosis, but the mechanism is unknown. Furthermore, agents that arrest the cell cycle have been shown to protect cells against TNF-mediated apoptosis. Gap junction expression also appears to protect cells against TNF-mediated apoptosis. These data

could indicate that TNF has a role in eliminating cells that are rapidly dividing or have reduced cell-cell communication such as transformed cells and malignant cells.

Numerous kinases are activated by TNF (Vanlint et al, 1992; Beyaert and Fiers, 1994). One of these, probably an unknown serine/threonine protein kinase appears to be important for protection of resistant cells from apoptosis (Beyaert et al, 1993). Furthermore, a protein phosphatase appears to be important for killing (Fletcher et al, 1987). It is likely that these two signals counteract each other with one signal regulating the action of the other. At this time, however, it is unknown if the protein kinase activates a protective signal by phosphorylation which is counteracted by the action of the phosphatase, or whether the phosphatase activates a lytic signal by dephosphorylation which is replaced by the action of the kinase. However, in the view that a protein kinase initiates the protective signal (Beyaert and Fiers, 1994), it is tempting to speculate that the phosphatase counter-regulates the target of the kinase.

TNF has been found to be a mediator of NC lytic activity (Ortaldo et al, 1986; Patek et al, 1987; Patek and Lin, 1991; Vanderslice and Collins, 1991). Antibodies against TNF can inhibit killing of susceptible target cells by NC effectors. Furthermore, both TNF- and NC-mediated killing have a four to six hour lag before efficient killing (Ortaldo et al, 1986; Patek et al, 1987). However, while target cells selected for resistance to TNF are resistant to NC in vitro, they do not produce tumors in normal mice as do target cells selected for resistance to spleen cells (Patek and Lin, 1991). While TNF plays a role in NC activity, it is not the sole effector molecule involved in NC activity and immune surveillance.

e. Objectives

Much of current research in NC activity is carried out using leukocytes from spleens, lymph nodes, or peripheral blood. Studies of the molecular biology of NC

effectors have proven to be difficult using such a heterogeneous population of cells. Cloned cell lines which have NC activity would be much more suited for studying the binding of effectors to targets. L10A2.J is a B cell lymphoma that has been found to kill the same targets as splenic NC and appear to express bona fide NC-activity (Collins et al, 1986). Other cloned B cell lines also have been shown to express TNF (Laskov et al, 1990; Ware et al, 1992).

There are two major objectives for the research presented here. First, we characterize a cloned NC effector, L10A2.J, which would enable future studies of the cellular and molecular biology of natural cytotoxicity. In addition, we used the clone to aid in the determination of the role of TNF in NC effector binding to targets.

Chapter 2. Materials and Methods

Mice.

Female BALB/c mice, 8 to 12 weeks old, were used.

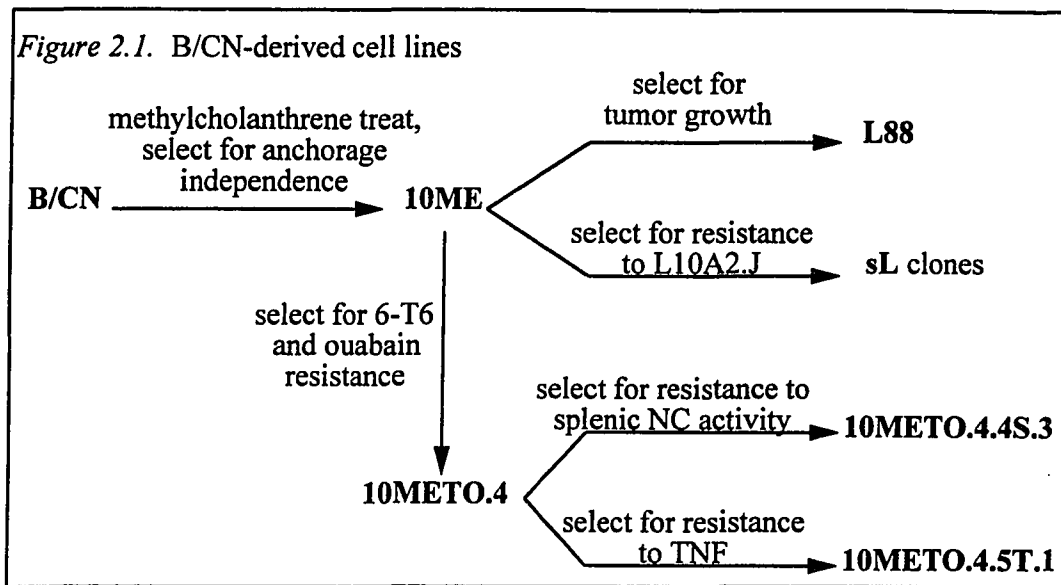
Target cell lines.

All cell lines were maintained at 37°C in humidified incubators with 7.5% CO₂.

All murine fibroblast cell lines were maintained in Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% bovine calf serum [BCS] (defined/supplemented; HyClone Laboratories, Inc., Logan, UT, lot no. 1115969), and penicillin (450 U/ml)/streptomycin (40 mg/ml) [pen/strep] (Sigma Chemical Co.) in tissue culture treated dishes. Maintained cell lines were passaged prior to confluency using 1X trypsin (Sigma), 0.04% ethylenediaminetetraacetic acid [EDTA] (Sigma) in Hank's BSS without Ca²⁺ and Mg²⁺ (400 mg/L KCl, 60 mg/L KH₂PO₄, 8 g/L NaCl, 48 mg/L Na₂HPO₄, 1 g/L glucose, 350 mg/L NaHCO₃; pH7.4). Two days prior to use, cells were placed in DMEM supplemented with 10% fetal calf serum [FCS] (HyClone lot no. 21511021), and pen/strep.

B/C-N is a cloned, continuous, nontransformed, nontumorigenic cell line derived from a BALB/c mouse embryo culture (Patek, personal communication). 10ME.HDA.5R.1 [10ME] is a cloned, methylcholanthrene-transformed variant of B/C-N which is tumorigenic in adult thymectomized, fetal liver-reconstituted [ATXFL] mice, but not tumorigenic in normal mice (Patek et al, 1978; Collins et al, 1981). L88.3 [L88] is a cloned tumorigenic cell line derived from 10ME; L88 was cloned following serial passage of 10ME, first in ATXFL then in normal mice (Collins et al, 1981). 10METO.4, a cloned, 6-thioguanine-ouabain-resistant variant of 10ME, is otherwise phenotypically indistinguishable from its parent, 10ME (Lin et al, 1983). 10METO.4.4S.3.3 [4S.3], is a variant of 10METO.4 cloned from a population selected in vitro for resistance to splenic

NC effectors (Patek et al, 1986). 10METO.4.5T.1 [5T1] is a variant of 10METO.4 cloned from a population selected in vitro for resistance to TNF- α (Patek and Lin, 1991). 10ME.sL.2, 10ME.sL.3, 10ME.sL.5, 10ME.sL.6, 10ME.sL.10, 10ME.sL.11, 10ME.sL.13, 10ME.sL.14, and 10ME.sL.17 [referred to as sL2, sL3, sL5, sL6, sL10, sL11, sL13, sL14, sL17, respectively] are variants of 10ME cloned from a population selected in vitro with L10A2.J as described below.



B cell effector cell lines.

The B cell lines ABPL 2.2, ABPL 4.3, L10A2.J, RAW 8.1, RAW 108, RAW 109, RAW 112.2, RAW 117.4, RAW 253.1, RAW 306.3, RAW 307F.1, RAW 309F.3, RAW 311.2, WR 13.2, WR 313.4, and 2PK3 were maintained in DMEM supplemented with 10% FCS, 2-mercaptoethanol (2ME) (50 mM) (Sigma), and pen/strep. The B cell lines ABPL 109.1, WEHI 231.2.1, and 18-48 were maintained in RPMI 1640 medium supplemented with 10% FCS, sodium pyruvate (1 mM), 2ME, and pen/strep.

L10A2.J is a spontaneous BALB/c B cell lymphoma (the lineage of L10A2.J is described in detail by Collins et al, 1986). Other B cell lines were cloned from populations transformed with either Ableson murine leukemia virus [AbMuLV] or

mineral oil and classified as pre-B, early B, or B cells (summarized in Table 5) (W.C. Raschke, The Salk Institute, personal communication; Raschke, 1980).

Table 2.1. Summary of the phenotypes of B cell lines

cell line	growth medium	method of transformation	B lineage type ^a	cell type ^a
ABPL 2.2	DMEM+	AbMuLV	preB	PL ^b
ABPL 4.3	DMEM+	AbMuLV	preB	PL
ABPL 109.1	RPMI+	AbMuLV	preB	PL
L10A2.J	DMEM+	spont.	B	lymphoma
RAW 8.1	DMEM+	AbMuLV	preB	
RAW 108	DMEM+	AbMuLV	preB	lymphoma
RAW 109	DMEM+	AbMuLV	preB	lymphoma
RAW 112.2	DMEM+	AbMuLV	preB	lymphoma
RAW 117.4	DMEM+	AbMuLV	preB	lymphoma
RAW 253.1	DMEM+	AbMuLV	preB	
RAW 306.3	DMEM+	AbMuLV	preB	lymphoma
RAW 309F.3	DMEM+	AbMuLV	preB	PL
RAW 311.2	DMEM+	AbMuLV	B	myeloma
WEHI 231.1	RPMI+	mineral oil	B	lymphoma
WR13.2	DMEM+	AbMuLV	preB	
WR313.4	DMEM+	AbMuLV	preB	
18-48	RPMI+	AbMuLV	preB	lymphoma
2PK-3	DMEM+	mineral oil	early	

^a (Raschke, 1980); W.C. Raschke, The Salk Institute, personal communication

^b PL= plasmacytoid lymphoma

^c DMEM+: DMEM, 10% FCS, pen/strep, 2ME

RPMI+: RPMI 1640, 10% FCS, pen/strep, 2ME, sodium pyruvate

Selection for L10A2.J-resistant variants of 10ME.

1 x 10⁵ 10ME cells were mixed with 1.6 x 10⁶ L10A2.J cells in 10 ml of DMEM supplemented with 10% FCS, 2ME, and pen/strep and incubated at 37° for 18 hours on a

rocking platform. Subsequently, the supernatant was removed and the plates were washed three times with phosphate-buffered saline [PBS] (133 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 100 mg/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 200 mg/L KCl, 200 mg/L KH_2PO_4 , 8 g/L NaCl, 1.15 g/L Na_2HPO_4 ; pH7.3) to remove floating or loosely adhering L10A2.J cells. The selected fibroblasts were allowed to grow for two passages in the above medium without 2ME in order to select against the L10A2.J. After four selection cycles, cells were cloned by limiting dilution.

The cloning procedure was as follows: 0.3 cells per well were placed in microtiter tissue culture dishes. Of the wells with growth, clones were selected from the wells with only one apparent colony. Clones were tested for sensitivity to lysis by spleen cells, L10A2.J, and recombinant human tumor necrosis factor- α [TNF] (a gift from L. Lin, Cetus Corp., Emeryville, CA). Lytic activity was assessed in a ^{51}Cr -release assay (see below). Clones that are phenotypically similar to 10ME are considered unselected or weakly selected.

Selection for variants of L10A2.J.

Variants of L10A2.J that do not bind to 10ME were produced by removal of the binding cells by their ability to stick to 10ME on a tissue culture dish. 2×10^6 10ME cells were plated on a 150 mm tissue culture dish containing DMEM supplemented with 10% FCS, 2ME, and pen/strep and incubated at 37° for 24 hours. The media were removed and 2×10^6 L10A2.J cells in 10 ml of DMEM supplemented with 10% FCS, 2ME, and pen/strep were added. This mixture was incubated at 37° for 30-60 min. on a rocking platform. Subsequently, the supernatant, with nonadhering L10A2.J cells, was plated on tissue culture dishes and incubated at 37° for 24 hours. After incubation, the L10A2.J in the resulting supernatant (which excludes residual 10ME that adheres to the tissue culture dish) were then plated on a petri dish. The selected L10A2.J [L10A2.J.sT]

were then expanded and reselected. Because the non-binding phenotype appears to be unstable, selection was performed for at least six consecutive days prior to the day of the assay.

Binding variants of L10A2.J.sT were similarly produced by selection with 10ME on tissue culture dishes. 2×10^6 10ME cells were plated on 150 mm tissue culture dishes containing DMEM supplemented with 10% FCS, 2ME, and pen/strep and incubated at 37° for 24 hours. The medium was removed and 2×10^6 L10A2.J.sT cells in 10 ml of DMEM supplemented with 10% FCS, 2ME, and pen/strep were added. This mixture was incubated at 37° for 30-60 min. on a rocking platform. Nonadhering L10A2.J.sT were removed in the supernatant and in three washes with PBS. Bound L10A2.J.sT were removed from adherent 10ME by vigorous agitation. These L10A2.J.sT were then plated on tissue culture dishes and incubated at 37° for 24 hours. The L10A2.J.sT in the supernatant (which excludes residual 10ME that adhered to the tissue culture dish) is then plated on a petri dish. The selected L10A2.J.sT [L10A2.J.sT(B)] were then expanded and reselected. Refer to chapter 5 for more detail.

The in vitro cytotoxic assay.

Target cells attached to tissue culture dishes were rinsed three times with PBS, then labeled for 80 min. with 0.1 mCi of ^{51}Cr in PBS per approximately 6×10^5 cells. Labeled cells were rinsed with serum-containing medium and detached from the dishes using 0.02% EDTA in Hank's BSS without Ca^{2+} and Mg^{2+} . 10^4 ^{51}Cr -labeled target cells were plated in each well of a 96-well microtiter tissue culture plate. Varying numbers of effector cells, varying concentrations of effector cell conditioned media, or human TNF were added to the targets with a final volume of 100 or 150 μl of supplemented RPMI medium (Epstein and Cohn, 1978). In some experiments that did not involve spleen cells, DMEM supplemented with 10% FCS, 2ME, and pen/strep was used. Spleen cell

effectors were from 8-12-week old normal BALB/c mice and were used at effector-to-target [E:T] ratios of 1.25:1, 5:1, 20:1, 80:1. B cell line effectors were titrated at E:T ratios of 0.25:1, 1:1, 4:1, and 16:1. B cell line conditioned media were prepared by 18-hour incubation of 8×10^5 cells/ml with or without 2×10^5 10ME cells/ml in supplemented RPMI medium. Supernatants were centrifuged 3 times to remove remaining cells and assayed at final concentrations of 50%, 13%, 3%, and 0.8% of the original supernatant. TNF was titrated at 0.01, 0.1, 1.0, and 10 U per well (1U is equivalent to the amount of TNF necessary to kill 50% of L929 targets; 1.3×10^7 U/mg). In experiments using polyclonal rabbit anti-mouse TNF antibody (Genzyme Corp., #IP-400, Cambridge, MA), 1 μ l of antibody was added (neutralizes approximately 1000 U of mouse TNF- α bioactivity) per well. ^{51}Cr release was determined at 6 hours for NK assays or 18 hours for NC assays. The percent specific ^{51}Cr release was calculated by the formula:

$$\text{percent specific lysis} = \frac{(\text{sample cpm} - \text{spontaneous release cpm}) \times 100}{(\text{total cpm} - \text{spontaneous release cpm})}$$

Each data point represents the average of triplicate experiments with the cpm of the triplicates within 10% of each other. Data shown are from representative experiments with spontaneous release of not more than 25%.

The effector cell binding assay

Target cells were suspended from tissue culture plates using 0.02% EDTA in Hank's BSS without Ca^{2+} and Mg^{2+} . 10^4 target cells per well were plated in DMEM with FCS, 2ME, and pen/strep and allowed to recover for 1 hour. Effector cells (L10A2.J or variants) were washed three times with PBS, then labeled for 80 min. with 0.1 mCi ^{51}Cr per approximately 1×10^6 cells in 1 ml PBS. ^{51}Cr -labeled effectors were washed with serum-containing media, then were plated at varying dilutions with a final volume of 100 or 150 μ l of supplemented RPMI medium. B cell line effectors were added at E:T ratios

of 1:1 or 4:1. A non-specific binding [NSB] control was also performed in wells without target cells. In experiments using polyclonal rabbit anti-mouse TNF antibody (Genzyme Corp., #IP-400, Cambridge, MA), 1 μ l of antibody was added (neutralizes approximately 1000 U of mouse TNF- α bioactivity) per well. After 45-60 min. of incubation, unbound cells were collected both from the supernatant and from two washes. Bound cells remained in the dishes and were lysed with detergent to ascertain the percentage bound to the targets. The percent specific effector binding was calculated by the formula:

$$\text{percent specific binding} = \frac{(\text{bound cpm} - \text{NSB cpm}) \times 100}{(\text{total cpm} - \text{NSB cpm})}$$

Each data point represents triplicate experiments. Data shown are from representative experiments with NSB of not more than 25%.

Tumorigenicity of cell lines

Cells were prepared in DMEM supplemented with 10% FCS, 2ME, and pen/strep. Subconfluent cells were removed from the dishes using 0.02% EDTA in Hank's BSS without Ca²⁺ and Mg²⁺. The resulting culture was washed three times with PBS and suspended in PBS at a concentration of 5 x 10⁷ cells per ml. 0.1 ml (5 x 10⁶ cells) was injected subcutaneously into normal syngeneic mice. Tumor formation was assessed weekly for 4 months.

Colony forming efficiency assay

L10A2.J cells were counted then diluted in DMEM supplemented with 10% FCS, 2ME, and pen/strep to a concentration of 9 cells/ml or 3 cells/ml. 100 μ l of the suspension was plated per well in a 96-well microtiter plate (i.e., 0.9 or 0.3 cells per well). 100 μ l of supplemented DMEM alone, medium with 10 U/ml TNF or 100 U/ml

TNF, or medium with 1000 cells/ml of mitomycin C-treated L10A2.J were added to wells. The mixtures were incubated four days at 37°C in 7% CO₂.

The number of wells with cells were counted and the colony forming efficiency was determined as follows:

$$\text{colony forming efficiency} = \frac{\text{wells with cells} \times 100}{\text{wells} \times \text{cells plated per well}}$$

Protein preparation

Proteins for sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE] were prepared as follows. Cells were rinsed three times in PBS, counted, and suspended in PBS at a concentration of 1 x 10⁸ cells per ml. 200 to 400 µl of SDS-PAGE sample buffer (5.3 ml distilled water, 1.2 ml 0.5 M 2-amino-2-(hydroxymethyl)-1,3-propanediol [Tris], pH6.8, 1 ml glycerol, 2 ml 10% SDS, 0.5 ml 2ME, 0.15 g bromphenol blue, 0.05 g phenol red) were added to each microfuge tube and allowed to boil for 5 min. in a water bath. An equal volume of cells in PBS was added to the microfuge tubes containing hot sample buffer and vortexed. The mixture was then boiled for 5 min. in a water bath. Unused samples were stored at -5°C and boiled for 5 min. prior to use.

Proteins for two-dimensional [2D] gel electrophoresis were prepared without SDS. Cells were rinsed three times in PBS and counted. The remaining PBS was removed and the cells were suspended in lysing solution (25 mM tris, 15 mM NaCl, 0.1% Triton X-100, pH 7.5) at a concentration of 1 x 10⁸ cells per ml. 200 µl aliquots were prepared in microfuge tubes. The suspension was boiled for 1 min. then frozen at -5°C. After thawing, the aliquots were diluted in 750 µl of first dimension sample buffer (9.5 M urea, 2% deionized Triton X-100, 5% 2ME, 1.6% 5/7 ampholyte, 0.4% 3/10 ampholyte). Unused samples were frozen at -5°C until needed.

2D-gel electrophoresis first dimension (isoelectric focusing)

2D gel electrophoresis involved the separation of proteins first by charge using isoelectric focusing [IEF] and then by size using SDS-PAGE. 5 ml first dimension gel solution (9.2 M urea, 3.8% acrylamide, 0.2% bisacrylamide, 2% deionized Triton X-100, 1.6% 5/7 ampholyte, 0.4% 3/10 ampholyte) was degassed and mixed with 15 μ l fresh 10% ammonium persulfate and 15 μ l N,N,N',N'-tetramethylethylenediamine [TEMED]. The mixture was poured into capillary tubes and allowed to polymerize. The gel-containing tubes were then placed into a tube gel apparatus with 20 mM NaOH in the upper reservoir and 10 mM phosphoric acid in the lower reservoir. 25-75 μ l of sample was added to each tube and 25-75 μ l of overlay buffer (9M urea, 0.05% Triton X-100, 0.8% 5/7 ampholyte, 0.2% 3/10 ampholyte, 1 mg/ml bromphenol blue) were overlaid over the sample. Samples were electrophoresed at 300 V for 15 min. then 400 V for 12-18 hours.

Gels were ejected from the capillary tubes and then treated for 5-10 min. in equilibration buffer (12.5 ml 0.5 M Tris, pH 6.8, 23 ml 10% SDS, 5 ml 2ME, 8 ml glycerol, 1.25 mg bromphenol blue, 49 ml distilled water). Tube gels were then laid on the stacking gel of a 12% SDS-PAGE slab (see next section).

SDS-PAGE

16 ml gel solution (29.2% acrylamide, 0.8% bisacrylamide) was mixed with 13.4 ml distilled water and degassed. The gel mixture was then mixed with 10 ml Tris, pH 8.8, 0.4 ml 10% SDS, 200 μ l fresh 10% ammonium persulfate, and 40 μ l TEMED. The separating gel mix was then poured into the glass plate apparatus, overlaid with distilled water, and allowed to polymerize. The remaining water layer was then removed and stacking gel mix (12.2 ml distilled water, 2.6 ml acrylamide, degassed, 5 ml 0.5 ml 0.5 M

Tris, pH 6.8, 0.2 ml 10% SDS, 150 μ l fresh 10% ammonium persulfate, 25 μ l TEMED) was then poured over the separating gel and allowed to polymerize.

25-150 μ l of sample (0.2 to 1 mg/ml protein) was added to each well. The gel is then placed in a chamber containing running buffer (0.3% Tris base, 2.16% glycine, 0.1% SDS) with a constant temperature of 10°C. A current of 15 mA per 7.5 mm gel was used until the tracking dye reached the separating gel at which point a current of 30 mA per 7.5 mm gel was used.

Gels were stained using Coomassie blue staining and/or the BioRad silver stain kit (BioRad #161-0443). The Coomassie blue staining procedure was performed as follows. The gel was placed in a tray, submerged in fixing solution (50% methanol, 10% acetic acid), and incubated with rocking for two hours at room temperature. Fixing solution was replaced with staining solution (50% methanol, 10% acetic acid, 0.5 g/L Coomassie brilliant blue R) and incubated with rocking for 15-30 min. at room temperature. The staining solution was removed and the gel was briefly rinsed in fixing solution. The gel was then incubated for one hour with rocking in destaining solution (5% acetic acid, 10% methanol). Destaining solution was replaced and the gel was further destained until bands are clearly distinguishable from the clear background. Gels were then rinsed with 7% acetic acid or distilled water and dried with vacuum dehydration and heat on Whatman filter paper.

Alternatively, silver staining was used. Gels were placed in a fixer 1 solution (10% acetic acid, 40% methanol) for one or more hours at room temperature, with rocking. This was followed by two 30 min. treatments with fixer 2 solution (5% acetic acid, 20% ethanol). Gels were then oxidized for 10 min. followed by six 10 min. rinses with distilled water. Subsequently, gels are treated with a silver reagent for 30 min. and then washed once with distilled water for 2 min. Gels are then exposed to developer for 30 sec. Developer is then exchanged and incubated for five or more minutes until protein

bands are easily distinguishable from the clear background. Excess developer is removed and 5% acetic acid is added to stop the development reaction. Silver-stained gels are dried as discussed above for Coomassie-stained gels.

Western blot hybridization

Unstained slab gels from SDS-PAGE are placed in a transfer sandwich adjacent to a nitrocellulose membrane wetted in transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol, pH8.3). Protein is electrophoretically transferred from the gel to the membrane in transfer buffer at 150 V for 1.5 hours at 5°C.

Membranes were rinsed for 10 min. in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) and then blocked with BLOTTO (5% non-fat dry milk)-TBS for one hour at room temperature with shaking. After two 5 min. rinses in TTBS (0.05% Tween-20, TBS), primary antibody (1:1000) in BLOTTO-TTBS is added and the membrane is incubated for 1.5 hours at room temperature with shaking. Rinses and similar treatments were done with biotinylated mouse anti-rabbit immunoglobulin monoclonal antibody (1:20,000), followed by streptavidin (1 µg/ml), and biotinylated horseradish peroxidase (2.5 µg/ml). Membranes were washed for 10 min. three times with TBS and then treated with ECL detection reagents (Amersham #RPN.2109). Excess reagent was removed and membranes were wrapped in Saran wrap plastic film.

Chemiluminescence was detected using Kodak XOMAT-LS X-ray film in an intensifying cassette. The X-ray film was exposed for 1.5 min., developed in Kodak GBX developer for 1.5 min., washed in water, and fixed in Kodak GBX fixer for 2 min.

mRNA preparation

All items coming in contact with RNA were diethyl pyrocarbonate [DEPC]-treated (0.1% DEPC was added to liquids; solids were exposed to 0.1% DEPC-water) and

autoclaved). Cells were rinsed three times with cold PBS and counted. Up to 3×10^8 cells were transferred to a polypropylene tube, centrifuged, decanted, and frozen in liquid nitrogen. mRNA was isolated from the cells by oligo(dT) cellulose microfuge spin columns using the FastTrack mRNA isolation kit (Invitrogen #K1593-02). mRNA bound to the oligo(dT) cellulose was eluted and precipitated. The precipitate was dissolved in elution buffer. A 1:100 dilution of this sample was then analyzed for the amount of RNA using the following formula:

$$[\text{mRNA}] = A_{260} \times (0.04 \mu\text{g/ml}) \times \text{dilution factor}$$

mRNA was also analyzed by formaldehyde gel electrophoresis (Raschke, personal communication). 3 volumes of RNA cooker (1 ml 10X HEN buffer (200 mM HEPES, 100 mM sodium acetate, 1 mM EDTA in DEPC-treated water), 5 ml deionized formamide, 18 μl 0.5 M EDTA, pH 7.4) was added to 2.5 μg mRNA and incubated for 4 min. at 65°C. After denaturation, the sample was incubated on ice and 2-3 μl of BPB dye (4 mg bromphenol blue, 10 ml glycerol, 10 ml DEPC-treated water) and 1 μl of ethidium bromide (5 mg ethidium bromide, 5 ml DEPC-treated water) was added. 0.75 g agarose was dissolved in 40 ml DEPC-treated water and 5 ml 10X HEN buffer. After 3-5 min. of cooling, 8 ml of formaldehyde was added and the gel mixture was poured into the gel tray. Samples were electrophoresed in 1X HEN buffer at 70V for 2 hours. Separated RNA was detected by UV transillumination.

Northern blot hybridization

Probes were labeled using the Biotin-21-dUTP labeling kit (Clontech #K1019-1). 1 μg of purified linear DNA was denatured and labeled using Klenow enzyme and biotin-labeled dUTP in the dNTP mixture.

RNA was treated with denaturing buffer (10 mM NaOH, 1 mM EDTA, in DEPC-treated water) and blotted onto a nylon membrane in a slot-blot manifold. The RNA was

then cross-linked to the membrane using 260 nM UV radiation for 3 min. The membrane was then placed in hybridization buffer (50% deionized formamide, 5X Denhardt's reagent (0.1% Ficoll, 0.1% polyvinylprolidone, 1% bovine serum albumin), 5X SSPE (0.9 M NaCl, 50 mM NaH₂PO₄, 5 mM EDTA) in DEPC-treated water) for blocking, 30 min., 42°C with shaking. Heat-denatured labeled probe was added and the hybridization was carried out 14 hours, 42°C with shaking. Excess probe was removed with three 10 min. washes using wash solution 1 (2X SSPE, 0.1% SDS in DEPC-treated water) at 42°C followed by four 15 min. washes using wash solution 2 (1X SSPE, 0.01% SDS in DEPC-treated water) at 60°C. The biotin-label was detected as per the western blot procedure (above).

Reverse transcriptase - polymerase chain reaction [RT-PCR]

mRNA was also analyzed using RT-PCR. 50-100 ng of mRNA was amplified using the StrataScript RT-PCR kit (Stratagene #200420) and TNF-specific amplimers (Clontech #K1019). This kit uses a two step protocol. In the first step, mRNA was converted to cDNA by RNase H⁻ reverse transcriptase. Then, the cDNA was amplified with specific primers by *Taq* DNA polymerase. Strand separation was carried out at 94°C for 45 sec., annealing was carried out at 60°C for 45 sec., and extension was carried out at 72°C for 2 min. 30 cycles were performed followed by a 7 min. 72° link period and a 4° soak period.

The resulting PCR product was analyzed in a 2% agarose gel with ethidium bromide staining. Electrophoresis was carried out in TBE (108 g Tris base, 55 g boric acid, 40 ml 0.5 M EDTA, pH 8.0) at 50 V for 2 hr. Separated DNA was detected by UV transillumination.

Chapter 3. Characterization of L10A2.J, a cloned NC effector

The first objective of this project is to characterize a cloned NC effector. A cloned NC effector will allow the study of the specific cellular and molecular properties of NC activity that is not possible with heterogenous spleen cell populations. In Chapters 3 and 4, we show that several cloned transformed B cell lines have NC activity. In particular, we focus on the BALB/C B cell lymphoma, L10A2.J. We find that L10A2.J is identical, in most characteristics, to splenic NC activity. These data have been published recently (Matsui and Patek, 1994).

a. L10A2.J has NC activity

NC activity is a cell-mediated cytolytic activity that is able to kill certain tumor targets in an 18-hr assay. Classically, spleen cells have been used as NC effectors. However, when dissecting the molecular mechanisms of NC activity, such a heterogenous population is difficult to utilize. It has been demonstrated that antibodies to a variety of cell surface determinants plus complement is unable to deplete splenic NC activity; among the antibodies tested are anti-H-2, -Thy-1, -Lyt-1, -L3T4, -surface immunoglobulin, -Qa-4, -Qa-5, -NK-1, and -asialo GM-1 antibodies (Stutman and Lattime, 1981). In contrast, positive selection using fluorescence activated cell sorting showed that leukocytes bearing H-2, Thy-1, L3T4, surface immunoglobulin, and Qa-5 antigens express NC activity (Stutman and Lattime, 1981). Recently, an antibody, NC-1.1, along with complement was found to be able to deplete NC activity from spleen cells (Smart et al, 1990a; Smart et al, 1990b). Taken together, these data indicate that there is a mixed population of cells including T cells, B cells, macrophages, and NK cells bearing NC activity and sharing the antigen recognized by NC-1.1. Because each of these cell types also have other properties, such a heterogenous population can not facilitate a

cellular and molecular study of NC activity. We describe here the characterization of L10A2.J as a cloned NC effector.

Spleen cell NC activity kills I-lines but not the N-lines from which they were derived, nor the C-lines derived from them. In this study, we use 10ME as an NC-sensitive I-line target, and B/CN and L88 as typical NC-resistant targets (Fig. 3.1). Typically, B/CN is more than 64-fold more NC-resistant than 10ME whereas L88 is more than 16-fold more NC-resistant (Fig. 3.1).

L10A2.J is a transformed B cell line which has been found to kill the same targets as splenic NC effectors (Collins et al, 1986). L10A2.J is able to kill NC-sensitive targets like 10ME and not NC-resistant targets like B/CN (Fig. 3.2). Again, B/CN targets are more than 64-fold more resistant than 10ME to L10A2.J-mediated killing. We will continue to use 10ME as a positive control and B/CN or L88 as negative controls for both the L10A2.J- and spleen cell-mediated killing experiments.

If L10A2.J is the same as splenic NC activity we would expect that L10A2.J itself would be a poor target for spleen cells. Indeed, L10A2.J is equally resistant to killing by L10A2.J and splenic NC activity (Fig. 3.3A and 3.3B, respectively). Furthermore, L10A2.J is more resistant than B/CN to killing by recombinant TNF, the putative lytic molecule of NC effectors (Fig. 3.3C). We thus assert that L10A2.J is a potential cloned effector for NC activity.

b. L10A2.J is not a natural killer, cytotoxic T lymphocyte, or antibody-dependent cytotoxic cell effector

The best known classes of cell-associated cytotoxic effectors are cytotoxic T lymphocytes [CTL], natural killer [NK] cells, antibody-dependent cell-mediated cytotoxicity [ADCC] cells, NC effectors, and activated macrophages. In characterizing

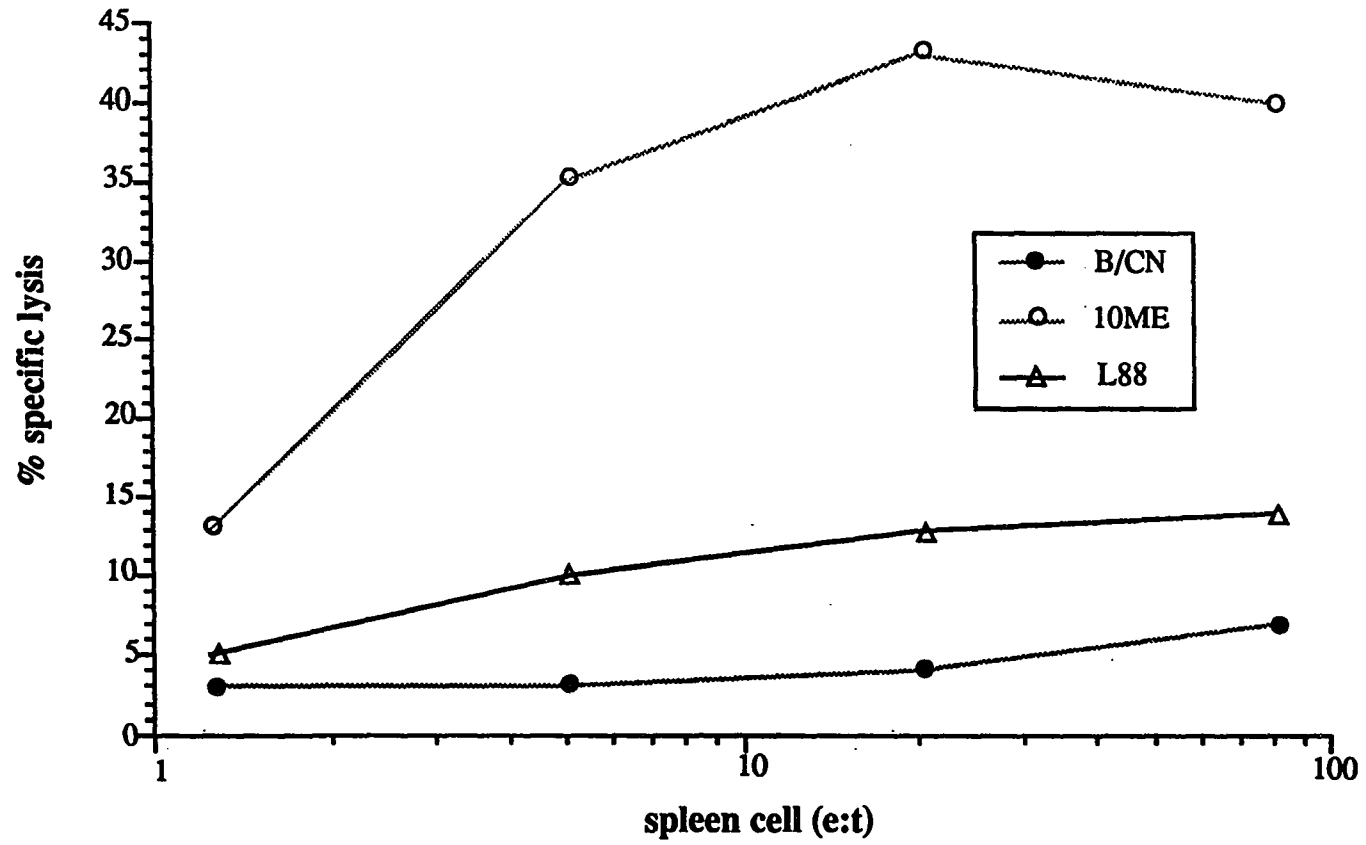


Figure 3.1. Spleen cells kill 10ME targets but not B/CN or L88 targets.

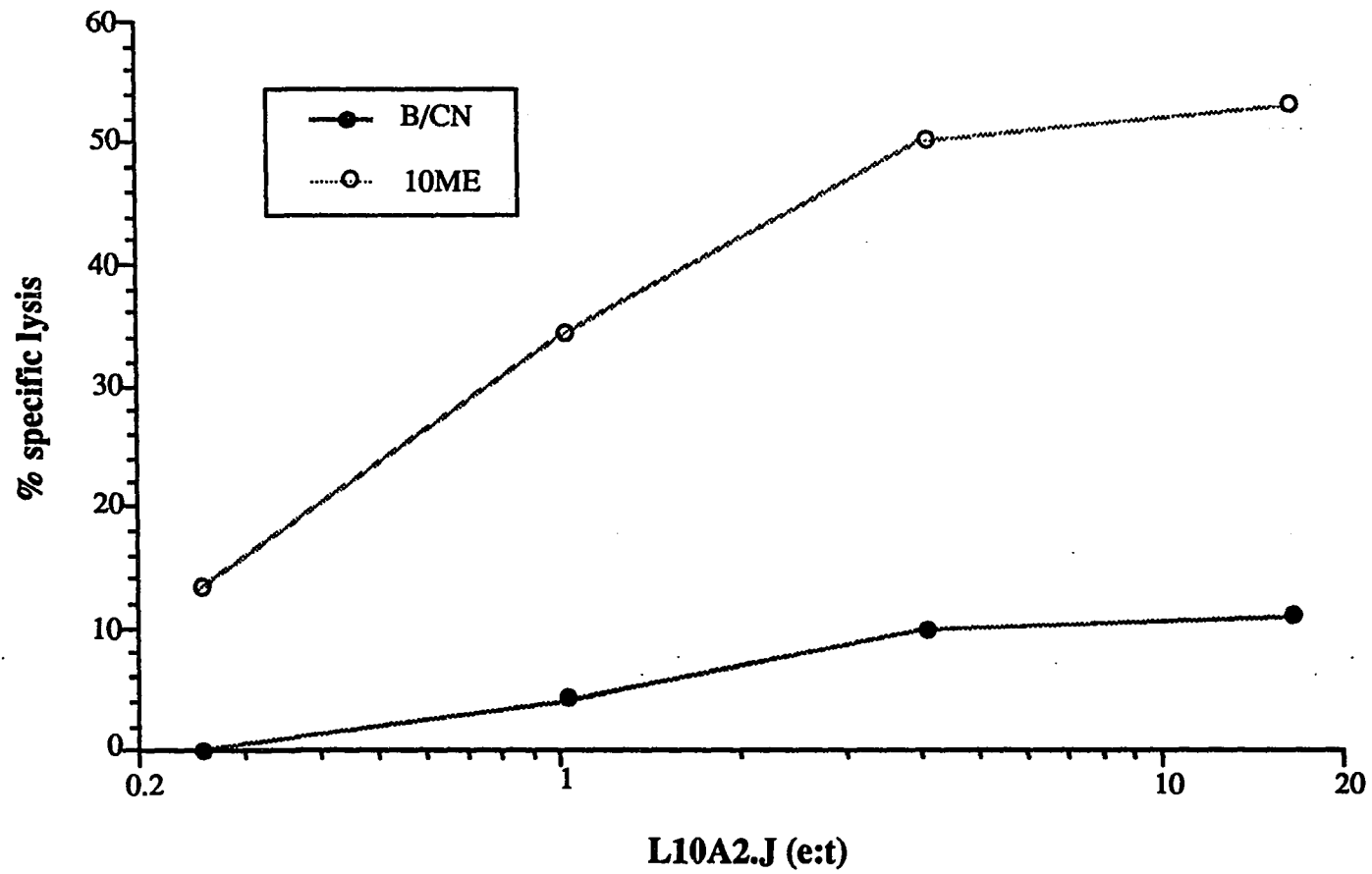


Figure 3.2. L10A2.J kills 10ME effectively, but not B/CN.

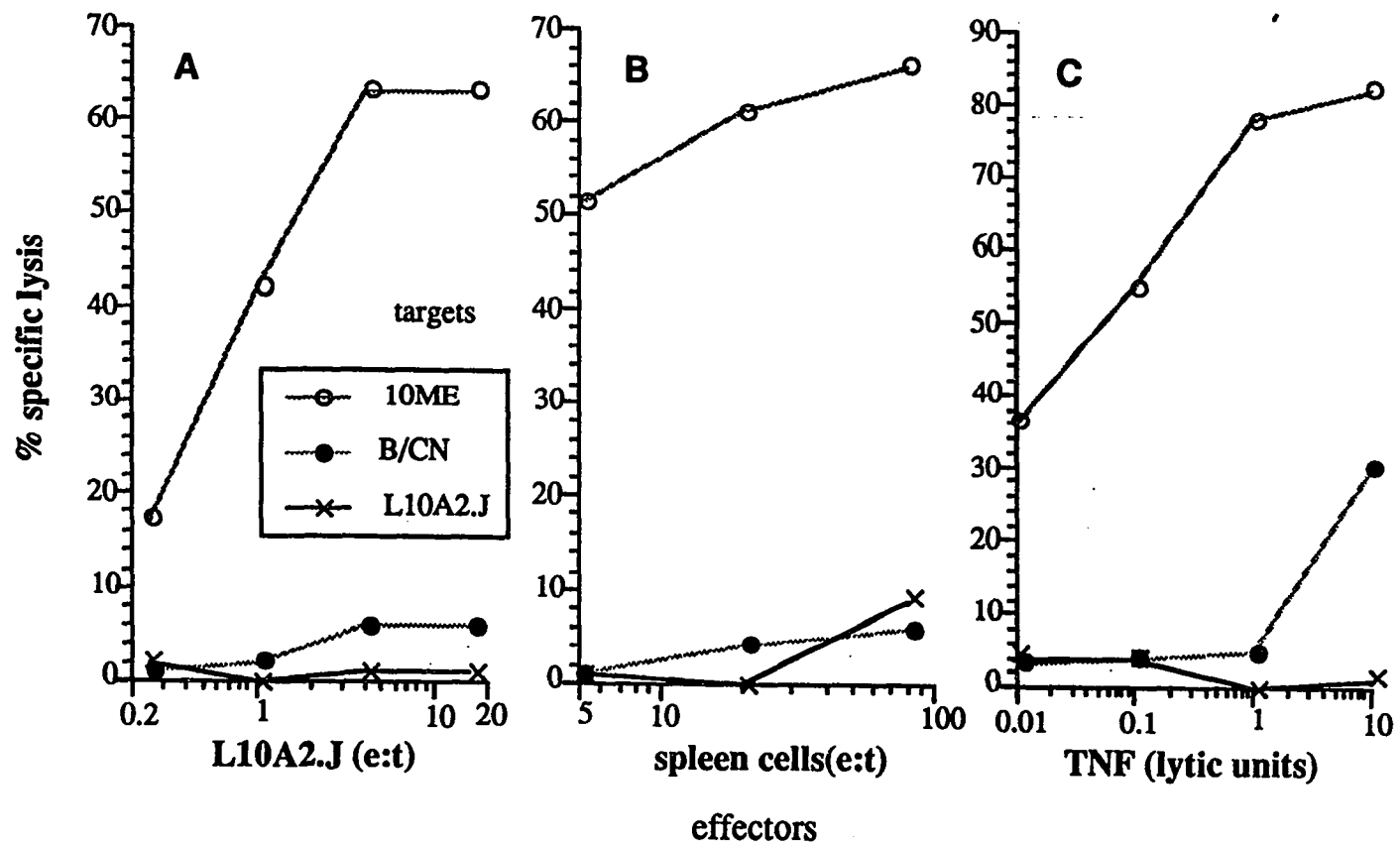


Figure 3.3. L10A2.J is not killed by L10A2.J, spleen cells, or TNF.

L10A2.J as an NC effector, it is necessary to distinguish these activities and to determine whether L10A2.J is also behaving like one of the other cytotoxic effectors.

Although NC activity and NK activity both belong to the category of NCMC and both appear to be mediated by heterogeneous populations, they show marked differences in their ranges of target cells and kinetics of killing (Lattime et al, 1981; Lattime et al, 1982; Lin et al, 1983). NC effectors kill targets such as Meth A, WEHI164, L929, 10ME, and 10CR which normally are adherent cell lines and form solid tumors in immune deficient mice. NK effectors kill targets such as Yac-1, K562, and Daudi which are normally nonadherent cell lines derived from murine or human lymphoid tumors in immune deficient mice. Furthermore, NK activity kills targets rapidly with efficient killing within six hours. This killing appears to be mediated primarily by perforin, a pore-forming protein that acts in a manner similar to complement, although other mechanisms have been proposed (Herberman et al, 1986). Conversely, NC activity kills targets after a lag of four to seven hours with maximum killing in 14-24 hours. This lag in killing is similar to that of TNF and is one piece of evidence arguing for a role of TNF in NC-mediated cytotoxicity (Ortaldo et al, 1986; Patek et al, 1987).

Analysis of the target cell range and kinetics of killing identifies L10A2.J as an NC rather than an NK effector (Fig. 3.4). Spleen cell populations contain NC and NK activity. Spleen cells are able to kill 10ME targets efficiently in 18 hours as an NC effector. They also exhibit NK activity in killing YAC-1 targets efficiently in 6 hours. TNF, the putative NC lytic molecule, kills 10ME efficiently in 18 hours, but does not kill YAC-1, the NK target. Likewise, L10A2.J kills 10ME efficiently in 18 hours, but does not kill YAC-1. Thus, L10A2.J appears to be an NC effector and not an NK effector.

Lymphotoxin [LT] (also referred to as TNF β) is a major molecular mediator of killing by CTL (Ferreri et al, 1992). LT has also been shown to exert its cytotoxic effect

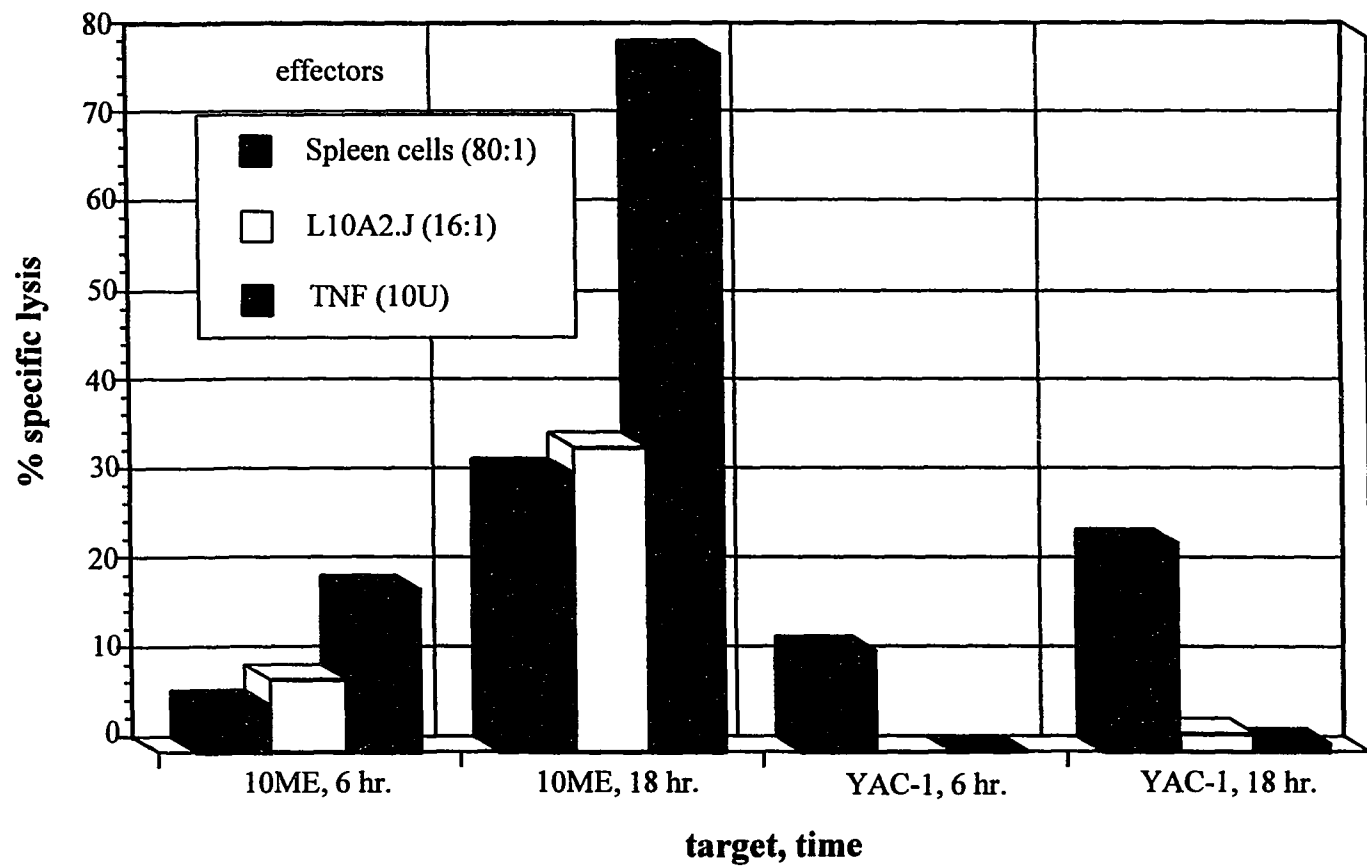


Figure 3.4. L10A2.J kills NC targets and not NK targets.

via the TNF receptor. Because the apoptotic effects induced by NC and CTL effectors are similar, we must determine whether L10A2.J is truly an NC effector or if it is behaving more like a CTL effector. Furthermore, some B cells were found to have a cytotoxic effect using LT (Laskov, 1990; Yamanaka and Karpas, 1989). CTL generally kill target cells in an antigen-dependent manner. NC effectors kill target cells but appear not to have an antigen-specific recognition system (Paige et al, 1978). Rather, their recognition repertoire appears to be broader and the recognition molecules are yet uncharacterized.

Concanavalin A [ConA] is a lectin that activates killing by allowing nonspecific binding of effectors to target cells. Splenic CTL can, thus, kill targets in an antigen-independent manner when ConA is present. Spleen cells, containing NC effectors as well as CTL, kill 10ME efficiently without ConA (Fig. 3.5A). Killing is increased with the addition of ConA, indicating the presence of CTL activity. Killing of 10ME by L10A2.J is unaffected by ConA (Fig. 3.5B). We interpret this to mean that L10A2.J does not have CTL activity. It is also possible that maximum killing has been achieved by L10A2.J without ConA. However, L10A2.J is a B cell and we do not expect T cell-like killing to occur due to a lack of necessary surface receptors such as the T cell receptor, CD3, and CD8.

Because L10A2.J is a B cell we must distinguish whether it kills targets by NC activity or by ADCC. In ADCC, specific antibody coats the target cell, subsequently, effector cells bind the antibody and exert a killing effect. LT has been implicated as an effector molecule for ADCC (Kondo et al, 1981).

We examined the possibility that L10A2.J might be an ADCC effector. The addition of anti-immunoglobulin antibodies does not decrease killing of 10ME by L10A2.J (Fig. 3.6). Because anti-immunoglobulin does not interfere with L10A2.J-mediated killing, we conclude that the latter is not due to a novel surface

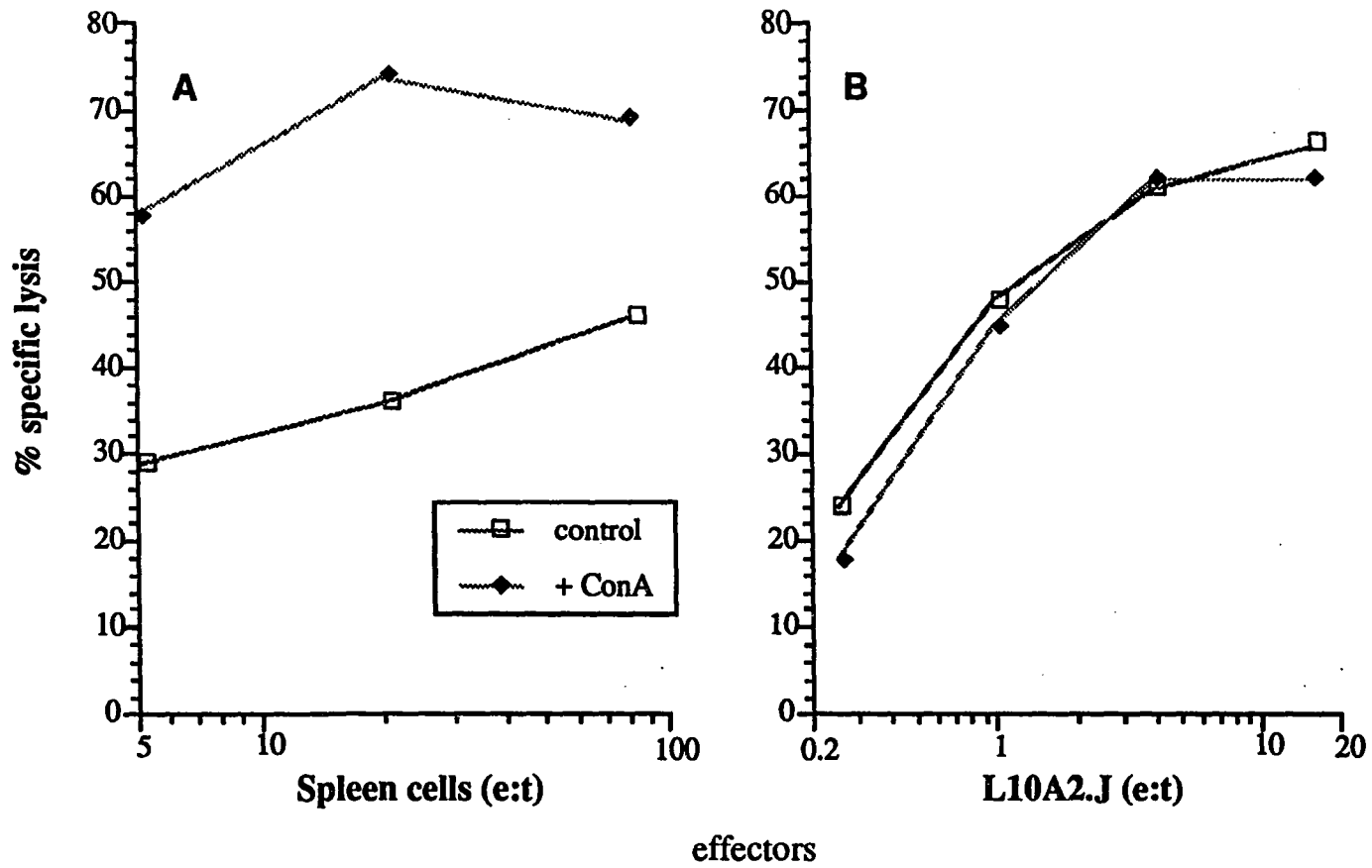


Figure 3.5. L10A2.J-mediated killing of 10ME targets is not affected by Concanavalin A, a CTL linking agent.

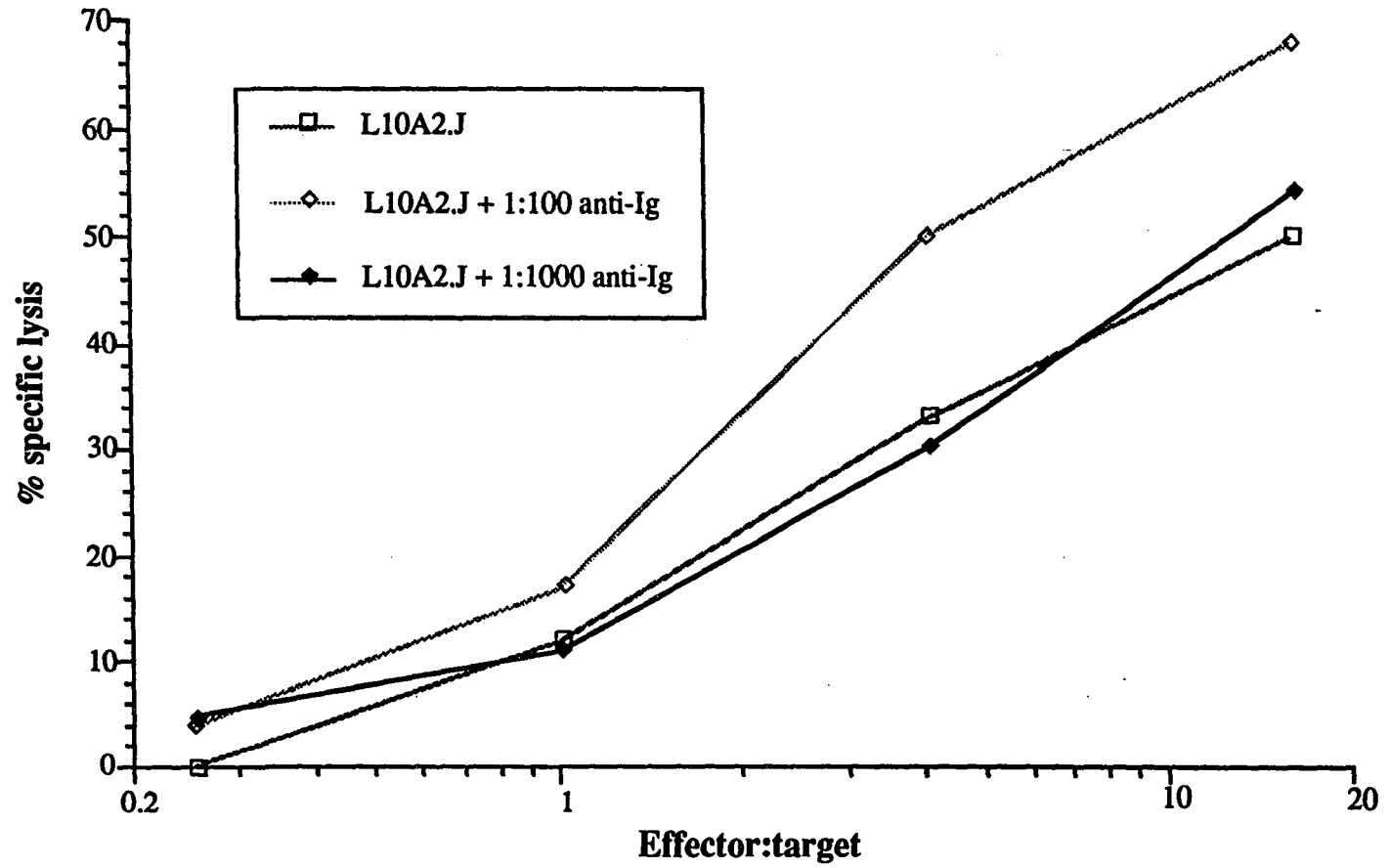


Figure 3.6. Anti-Ig antibody does not decrease killing of 10ME targets by L10A2.J.

immunoglobulin-mediated ADCC. Taken together, the data indicate that L10A2.J is an NC effector rather than an NK, CTL, or ADCC effector.

c. L10A2.J kills I-lines, but not C-lines

In the model of tumorigenesis proposed by Collins and Patek, cells progress to the cancer phenotype from a normal phenotype via an immunosensitive intermediate cell type (Patek et al, 1978; Collins et al, 1982). N-cell lines have a normal phenotype, I-lines have an intermediate transformed phenotype in that they form tumors in immune deficient mice only, and C-lines have a transformed phenotype and are able to form tumors in normal mice. It was determined that splenic NC cells were able to kill I-lines, but not N-lines or C-lines. Presumably, N-lines have a normal means of resisting NC activity which is compromised upon transformation. C-lines have gained the ability to escape immune surveillance by NC activity, and are thus resistant. L88 is a C-line derived from 10ME by growth as a tumor in ATXFL mice followed by growth as a tumor in normal mice (Fig. 3.7A). L88 is resistant to NC-, L10A2.J-, and recombinant TNF-mediated killing (Fig. 3.7B, 3.7C, 3.7D, respectively). L88 is more than 16-fold more resistant than 10ME to spleen cell-mediated killing, approximately 16-fold more resistant than 10ME to L10A2.J-mediated killing, and more than 100-fold more resistant than 10ME to TNF-mediated killing. Thus, L10A2.J kills *in vivo* selected C-lines similar to splenic NC or TNF.

10METO.4 is a cell line derived from 10ME which was selected for resistance to the metabolic inhibitor ouabain, but in all other respects it is phenotypically equivalent to 10ME (Patek et al, 1986). 10METO.4.4S.3 is a variant of 10METO.4 which was selected *in vitro* for resistance to spleen cell-mediated NC lysis (Patek et al, 1986). 10METO.4.5T.1 is a variant of 10METO.4 which was selected *in vitro* for resistance to TNF (Patek et al, 1991). Both 10METO.4.4S.3 and 10METO.4.5T.1 are resistant to splenic NC effectors as well as L10A2.J and recombinant TNF (Fig. 3.8B, 3.8C, 3.8D,

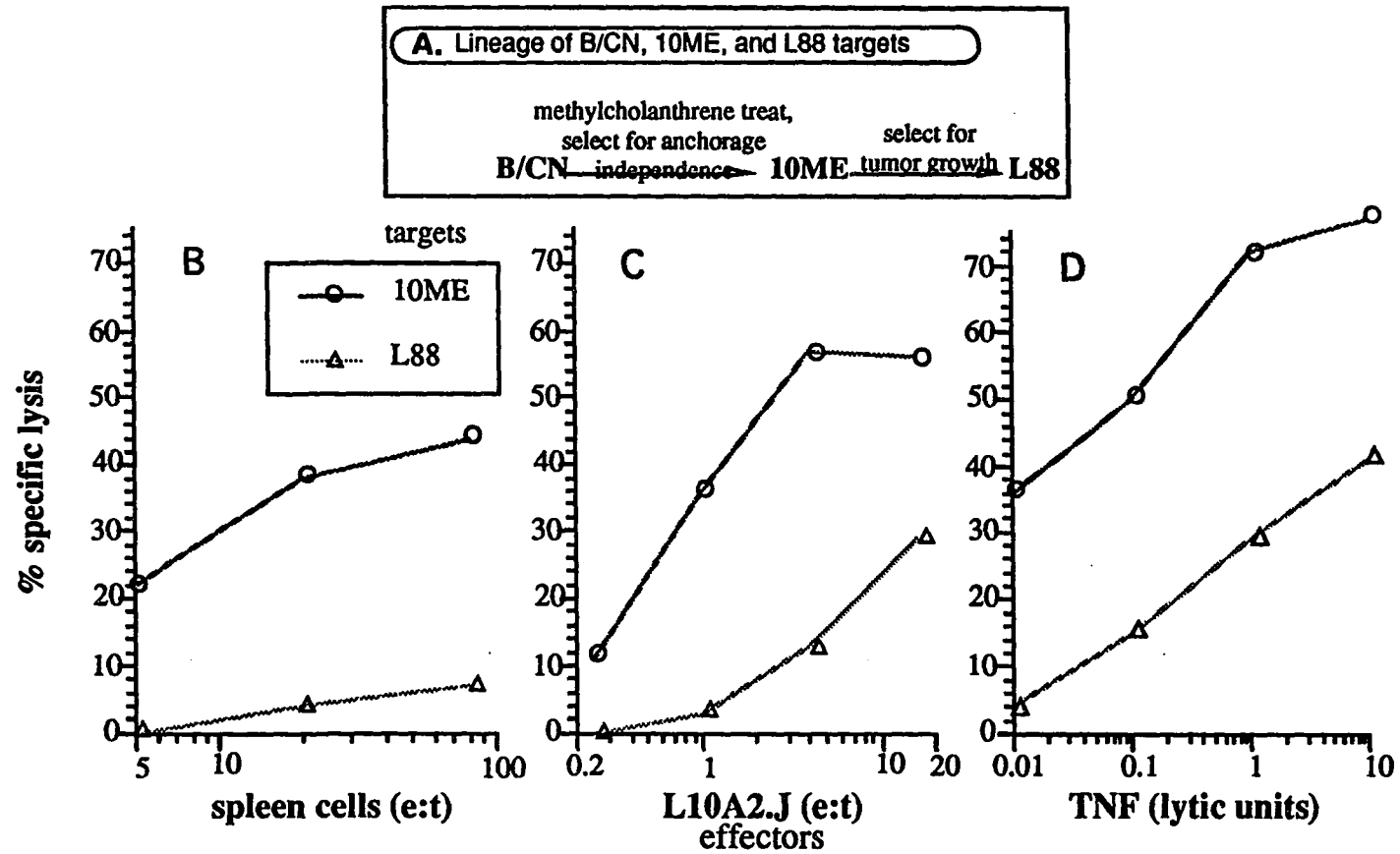


Figure 3.7. L10A2.J does not effectively kill L88, a variant of 10ME selected in vivo for the ability to form tumors.

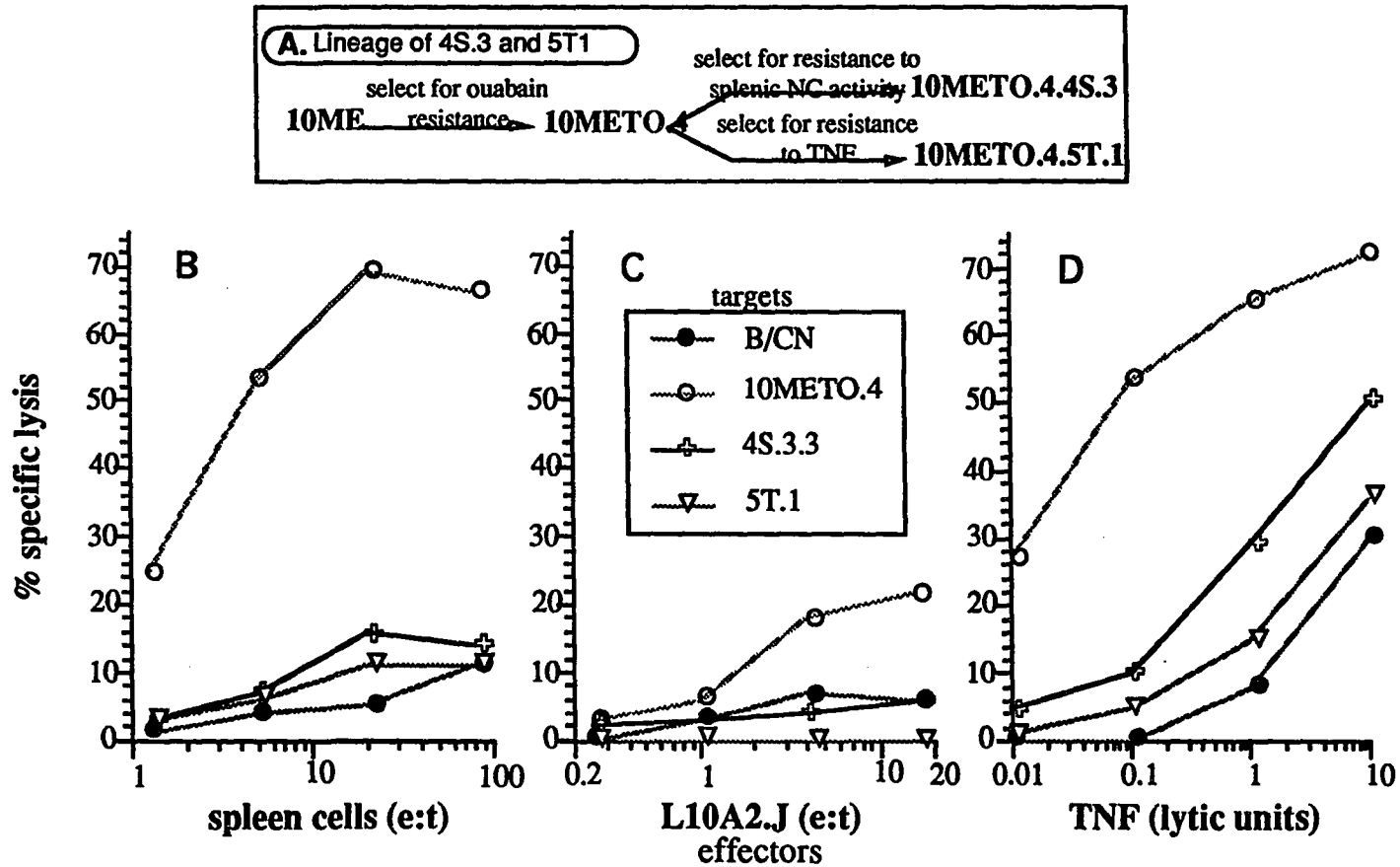


Figure 3.8. L10A2.J does not effectively kill 4S.3, a variant of 10ME selected in vitro for resistance to spleen cells, and 5T.1, a variant of 10ME selected in vitro for

respectively). Therefore, selection in vivo for tumorigenesis, in vitro for resistance to splenic NC activity, or in vitro for resistance to TNF selects for resistance to splenic NC, L10A2.J, and TNF.

d. selection of cell lines resistant to L10A2.J

To prove that L10A2.J is an NC effector it was important to determine whether cells selected by L10A2.J are equivalent to those selected in vivo for tumorigenesis, in vitro for resistance to spleen cells, and in vitro for resistance to TNF. 10ME was selected in vitro for resistance against L10A2.J-mediated killing. The resulting population of cells was then cloned, and the clones were expanded. A portion of each clone was frozen, and a portion was tested for sensitivity to spleen cell- and L10A2.J-mediated killing (Table 3.1). Of 20 clones nine were eventually tested. The other 11 clones either failed to grow, grew too slowly to be useful, or were lost due to contamination. Of the nine clones tested, 10ME.sL.2, 10ME.sL.6, and 10ME.sL.17 were resistant to L10A2.J (i.e., selected), the remaining clones were either unselected (10ME.sL.5, 10ME.sL.10, 10ME.sL.11, and 10ME.sL.13) or partially selected (10ME.sL.3 and 10ME.sL.14) (Tables 3.2 and 3.3). The unselected cell lines, such as 10ME.sL.10, were partially resistant to spleen cells and L10A2.J lytic activity but remained sensitive to TNF (Fig 3.9A, 3.9B, 3.9C, respectively). The selected cell lines, such as 10ME.sL.6, were spleen cell- and L10A2.J-resistant, but only partially TNF-resistant. Resistance to spleen cells and L10A2.J was comparable to that of B/CN, while resistance to TNF was approximately 10-fold more than that of 10ME. Cells selected by L10A2.J are therefore similar to those selected by NC, TNF, or selected for tumorigenicity. However, because 10ME.sL.6 was only slightly resistant to TNF, apparently L10A2.J is only weakly selective for TNF resistance. This suggests that NC and TNF resistance are not necessarily the same phenomenon and, therefore, that NC is more than just a form of TNF lytic activity.

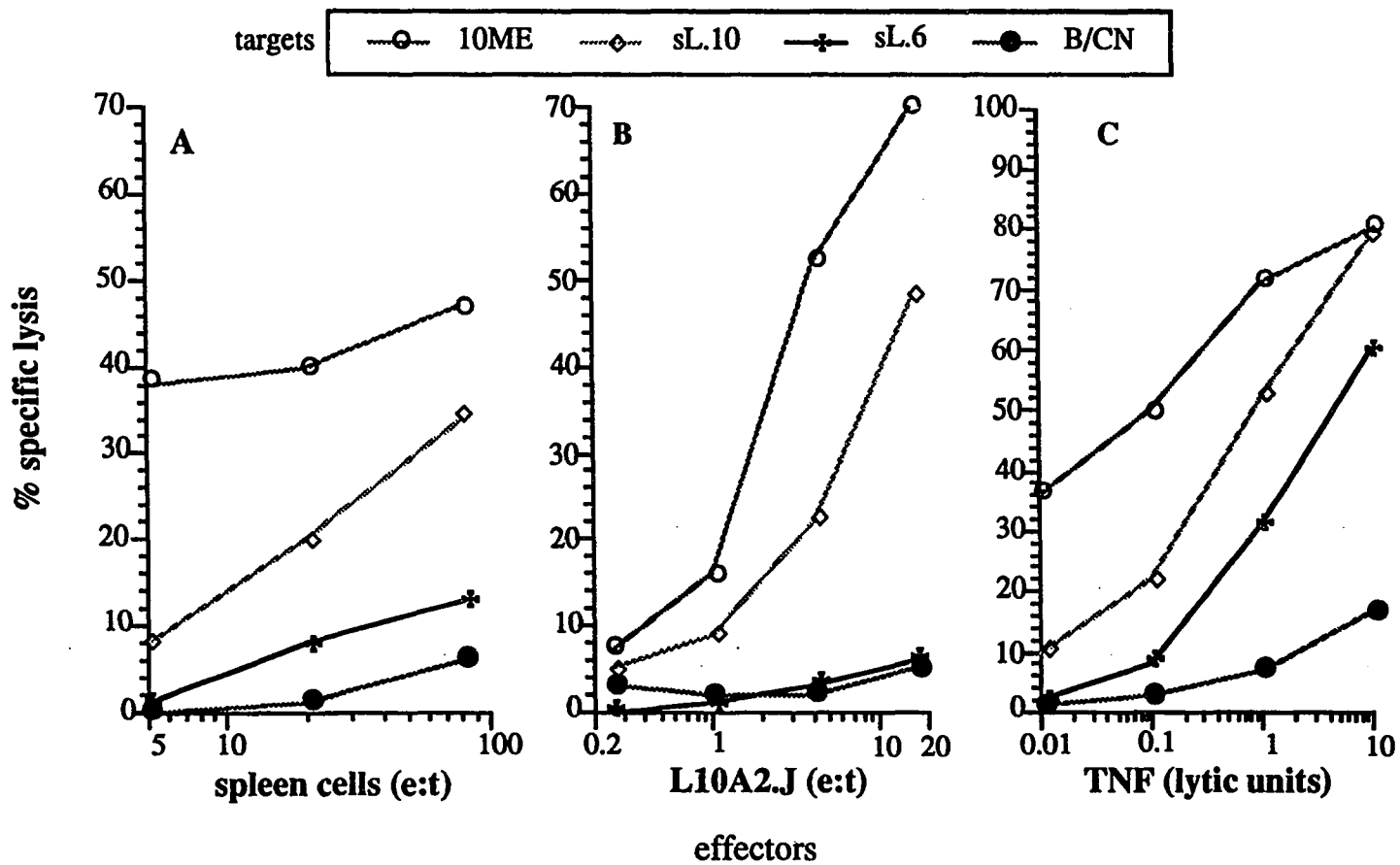


Figure 3.9. L10A2.J-selected 10ME targets are resistant to spleen cells and

Table 3.1. Cloning of L10A2.J-selected 10ME

#	morphology	growth	spleen cells			L10A2.J		
			5:1	80:1	s/r ^a	1:4	4:1	s/r
1	very spread out, flat	very slow						
2	10ME-like	moderate						
3	spread out, flat	slow						
4	slightly spread	moderate	36	58	s	2	36	s
5	slightly spread	moderate	42	39	s	9	32	s
6	slightly spread	moderate	3	25	r	0	4	r
7	(died)							
8	slightly spread	moderate	8	9	r	0		(r)
9	very spread out, flat	hardly						
10	10ME-like	fast	19	48	s	4	43	s
11	slightly spread	moderate						
12	10ME-like	fast	32	73	s	8	51	s
13	spread out, spindly	slow	22	50	s	1		(?)
14	spread out, flat	slow						
15	(died)							
16	10ME-like	moderate	10	47	s	2	16	r
17	slightly spread	fast	4	16	r	4	11	r
18	spread out, spindly	slow						
19	(died)							
20	(died)							
	(10ME control)		38	49	s	12	41	s
	(L88 control)		9	11	r	4	11	r

^a s = sensitive, r = resistant, () indicate insufficient data

Table 3.2. L10A2.J-resistant cell lines are NC resistant (Expt. 1)

target	spleen cells				L10A2.J					TNF				
	5:1	20:1	80:1	s/r ^a	1:4	1:1	4:1	16:1	s/r	0.01	0.1	1	10	s/r
B/CN	0	1	6	r	3	2	2	5	r	1	3	7	17	r
10ME	38	40	47	s	7	16	52	70	s	36	50	71	80	s
sL3	7	14	22	i	3	7	19	36	s	14	22	47	69	i
sL6	1	8	13	r	0	1	3	6	r	2	8	31	60	i
sL10	8	20	34	s	5	9	22	48	s	10	22	52	79	s
sL11	14	29	48	s	2	5	16	32	s	14	33	60	79	s
sL13	4	19	32	s	4	5	7	25	s	9	23	65	65	s

^a s = sensitive, r = resistant, i = intermediate

Table 3.3. L10A2.J-resistant cell lines are NC resistant (Expt. 2)

target	spleen cells				spleen cells					TNF				
	5:1	20:1	80:1	s/r	1:4	1:1	4:1	16:1	s/r	0.01	0.1	1	10	s/r
B/CN	2	3	6	r	4	5	10	9	r	5	8	16	27	r
10ME	17	31	39	s	23	38	69	59	s	57	69	68	70	s
sL2	4	9	21	r	6	7	17	22	r	39	82	109	108	s
sL5	16	26	32	s	9	16	34	41	s	35	55	56	61	s
sL14	10	17	26	i	10	26	41	37	s	29	50	54	65	s
sL17	2	0	4	r	0	0	3	3	r	55	60	60	70	s

^a s = sensitive, r = resistant, i = intermediate

I-lines such as 10ME form tumors in immune-deficient mice but not in normal mice (Lin et al, 1985). C-lines are able to form tumors in both immune deficient and normal mice. Cells selected for tumorigenicity, such as L88, and cells selected for resistance to spleen cells in vitro, such as 10METO.4.4S.3, form tumors in normal mice (Patek et al, 1986; Lin et al, 1985). However, cells selected in vitro for resistance to TNF such as 10METO.4.5T.1 do not form tumors in normal mice (Patek and Lin, 1991).

We assessed the in vitro tumorigenicity of six cell lines selected for resistance to L10A2.J (Fig. 3.10). 5×10^6 cells were subcutaneously injected into normal BALB/c mice. Mice were checked weekly for four months and positive tumors were tallied. Those cell lines that were NC- and L10A2.J-resistant (10ME.sL.2, 10ME.sL.6, and 10ME.sL.17) were also tumorigenic. Those cell lines that remained sensitive to splenic NC activity (10ME.sL.10, 10ME.sL.11, and 10ME.sL.13) showed no tumorigenicity.

Therefore the degree of tumorigenicity and resistance to splenic NC- and L10A2.J-mediated lysis of L10A2.J-selected cells such as 10ME.sL.6 correlate with that of cells selected for resistance to spleen cells in vitro such as 10METO.4.4S.3 and that of cells selected for tumorigenicity in vivo such as L88 (summarized on Table 3.4). The fact that TNF-selected cells such as 10METO.4.5T.1 do not form tumors although they are NC-, L10A2.J, and TNF-resistant suggests that NC activity involves TNF as well as other factors (summarized on Table 3.4). This factor could be a secondary lytic factor, another cell signal, a recognition determinant, or a binding determinant. It is possible that a different signal is exerted by membrane-associated TNF than secreted TNF, and that this will account for the difference in selection by TNF and that of selection by spleen cells or L10A2.J cells. A similar effect has recently been described for LT (Crowe et al, 1994).

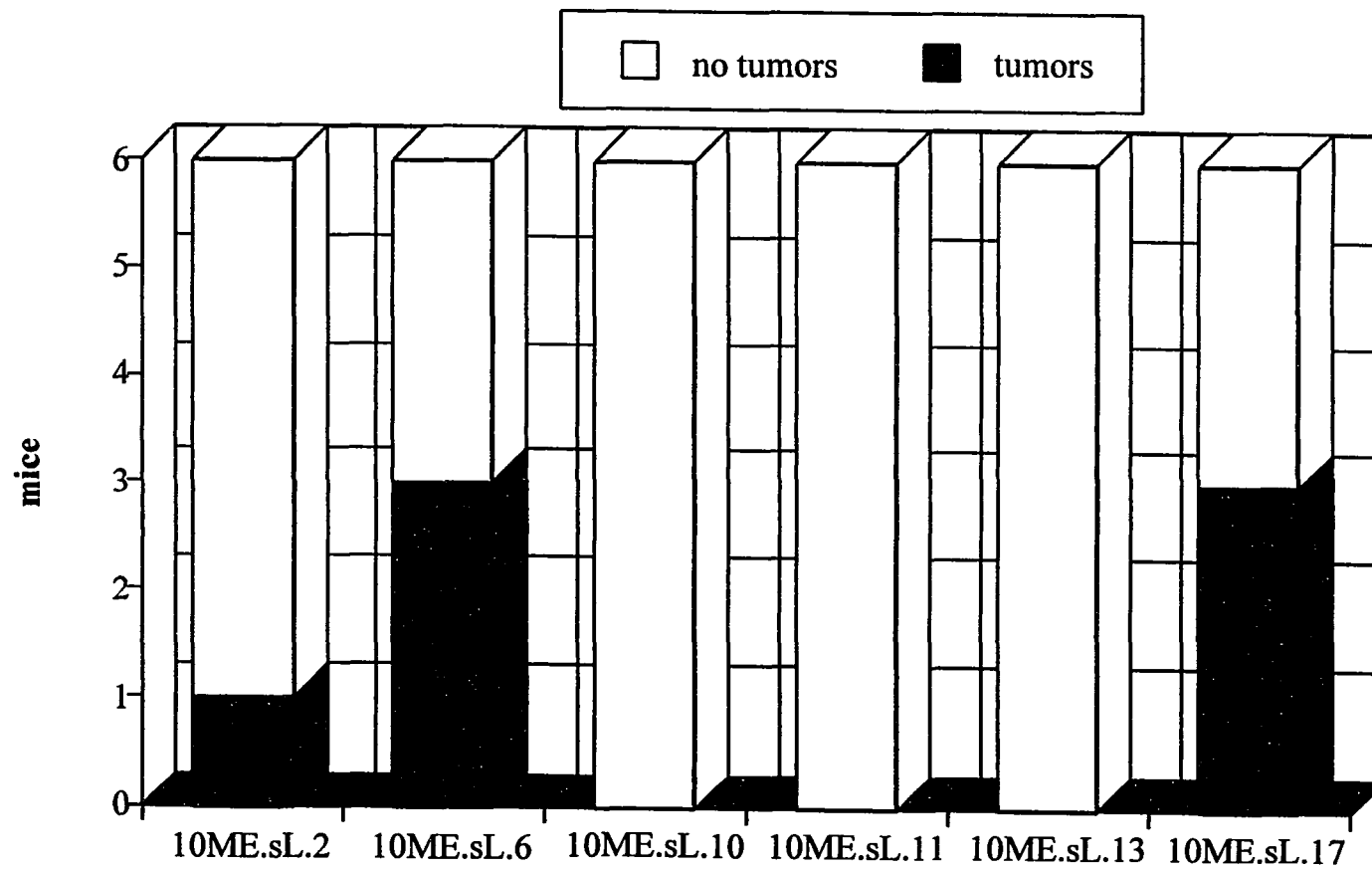


Figure 3.10. L10A2.J-selected 10ME clones cause tumors in normal BALB/c mice.

TABLE 3.4. Summary of the phenotypes of L10A2.J-selected variants of 10ME and other B/CN-derived cell lines

target cell line	parent line	selection	sensitivity to			tumorigenic in normal mice
			NC	L10A2.J	TNF	
B/CN	(primary)		r ^a	r	r ^b	- ^c
10ME	B/CN	anc. indep [†]	s ^a	s	s ^b	- ^{c*}
L88	10ME	tumor	r ^a	r	r ^b	+ ^c
10METO.4	10ME	ouabain	s ^d	s	s ^d	- ^{d*}
4S.3.3	10METO.4	splenic NC	r ^d	r	r ^d	+ ^d
5T.1	10METO.4	TNF	r ^e	r	r ^e	- ^e
sL.2	10ME	L10A2.J	r	r	s	+
sL.3	10ME	L10A2.J	i	i	s	n.d.
sL.5	10ME	L10A2.J	i	i	s	n.d.
sL.6	10ME	L10A2.J	r	r	i	+
sL.10	10ME	L10A2.J	i	i	s	-
sL.11	10ME	L10A2.J	i	i	s	-
sL.13	10ME	L10A2.J	i	i	s	-
sL.14	10ME	L10A2.J	i	i	s	n.d.
sL.17	10ME	L10A2.J	r	r	i	+

^a Collins et al, 1981

^b Patek et al, 1987

^c Lin et al, 1985

[†] anchorage independence

^d Patek et al, 1986

^e Patek and Lin, 1991

* is tumorigenic in immunodeficient mice

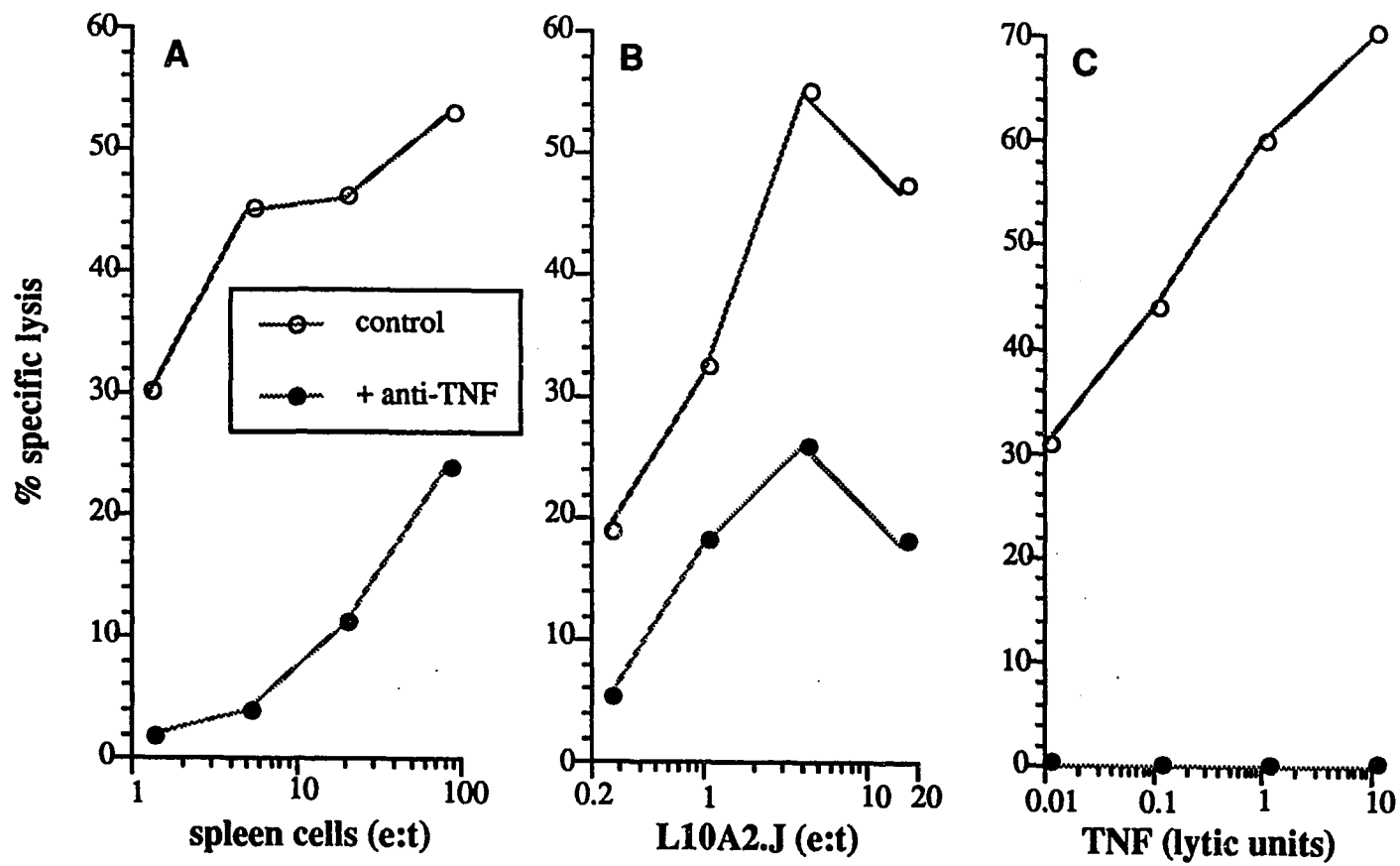


Figure 3.11. Antibodies against TNF inhibits killing of 10ME targets by splenic and L10A2.J natural cytotoxicity.

e. L10A2.J-mediated cytotoxicity involves TNF

Studies have indicated that TNF is the lytic effector molecule for NC effectors (Ortaldo et al, 1986; Patek et al, 1987). Both NC and TNF kill similar targets and have a lag before killing. Antibody against TNF will inhibit killing of 10ME by spleen cells, L10A2.J, or TNF (Fig. 3.11A, 3.11B, 3.11C). Thus, L10A2.J - mediated killing involves TNF, as do splenic NC effectors. The fact that anti-TNF antibody blocks 100% of the TNF lytic activity but less than 100% of the splenic NC activity or L10A2.J activity again suggests that the cell-mediated activities comprise TNF plus other factors.

f. Conclusions

It appears that L10A2.J is a cloned NC effector. L10A2.J exhibits characteristics of NC activity and not NK, CTL, or ADCC activities. Also, L10A2.J kills NC-sensitive targets such as 10ME and 10METO.4, and not NC-resistant targets such as L88, 10METO.4.4S.3, and 10METO.4.5T.1. This correlates with splenic NC activity. Additionally, L10A2.J-selected cells are resistant to spleen cell-, L10A2.J-, and TNF-mediated killing and are tumorigenic. Finally, antibody against TNF reduces killing by L10A2.J similar to the reduction in killing by spleen cells (see summary of the data in table 3.5). Overall, the results indicate that L10A2.J can be used as a cloned NC effector.

Table 3.5. Comparison of splenic NC and L10A2.J

characteristic	splenic NC	L10A2.J
kills NC targets (ex: 10ME)	+	+
kills NK targets (YAC-1)	-	-
kills in vivo selected targets (L88)	-	-
kills spleen cell-selected targets (10METO.4.4S.3)	-	-
kills L10A2.J-selected targets (10ME.sL.6)	-	-
selected cells cause tumors in normal mice	+(4S.3)	+(sL.6)
inhibited by anti-TNF antibody	+	+

Chapter 4. Other B cell lines mediate NC activity

Because L10A2.J mediates NC-like activity, and several B cell lines are known to secrete TNF or LT (Laskov et al, 1990; Ware et al, 1992), we investigated other B cell lines and found that they too have NC-like activity. Furthermore, this activity is mediated by a cell-associated mechanism similar to that found in L10A2.J and splenic NC effectors. Thus, certain B cells may have NC-like cytotoxic activity that is independent of their more commonly known antibody-producing activity.

The information presented here has recently been published (Matsui and Patek, 1994).

a. Several Pre-B and B cell lines have NC-like activity

Because L10A2.J has NC-like activity, we screened other cloned B cell lines to determine whether they express similar properties. Several pre-B and B cell lines were tested for cytolytic activity against 10ME. Figure 4.1 demonstrates the range of cytolytic activities observed (complete data are described on Tables 4.1, 4.2, and 4.3; summarized in Table 4.4). Cell lines such as RAW 253.1 and RAW 311.2 had no significant cytolytic activity against 10ME targets. RAW 309F.3 and WR313.4 had minimal cytolytic activity. Most of the cell lines tested had a significant amount of cytolytic activity. The cytotoxic activities of RAW 8.1, RAW 109, RAW 117.4, WR 13.2, and 18-48 were comparable to that of L10A2.J, while the cytotoxic activities of ABPL 2.2, ABPL 109.1, RAW 108, RAW 306.3, and WEHI 231.1 were significantly less than that of L10A2.J. A few lines such as ABPL4.3 and 2PK-3, have more lytic activity than L10A2.J.

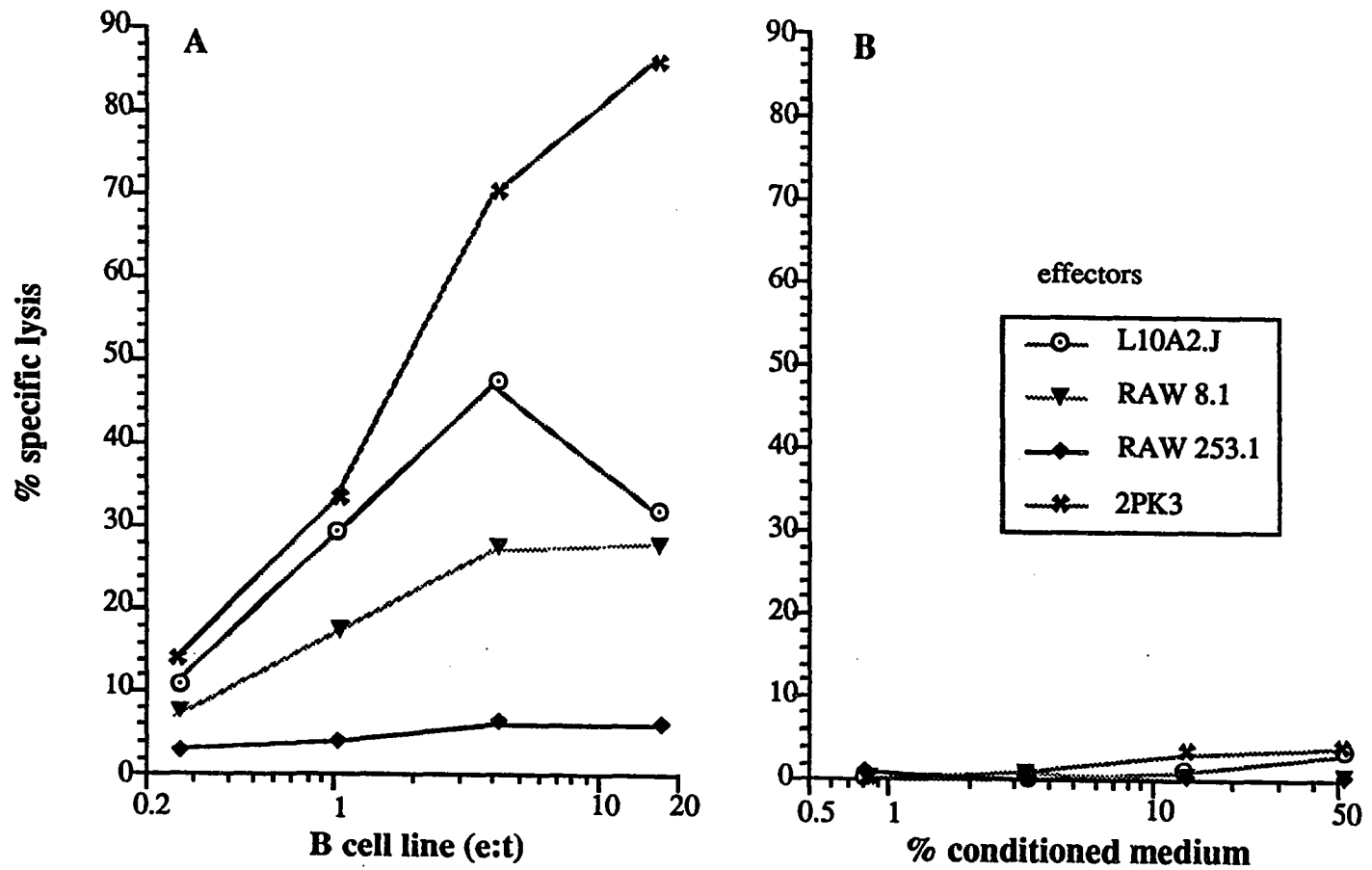


Figure 4.1. Several B cell lines have NC activity against 10ME targets

Table 4.1. B cell effectors have cell-mediated natural cytotoxic activity (expt 1)

effector	target	LPS-treated effectors								conditioned media				conditioned media made in the presence of 10ME			
		1:4	1:1	4:1	16:1	1:4	1:1	4:1	16:1	0.8%	3.1%	13%	50%	0.8%	3.1%	13%	50%
L10A2.J	B/CN	4	6	11	9	0	5	11	12	2	2	3	4	3	2	2	2
L10A2.J	10ME	19	38	54	52	27	44	61	59	3	3	2	0	0	0	0	0
WEHI231.1	10ME	4	11	23	30	4	7	16	19	0	0	0	0	0	0	0	0
ABPL4.3	10ME	45	61	70	91	50	65	72	71	0	5	13	1	1	14	12	20
RAW112.2	10ME	0	9	20	34	15	22	21	27	0	0	0	0	0	0	0	0
RAW306.3	10ME	2	7	16	28	2	11	22	28	0	0	0	0	0	0	0	0
RAW309F.3	10ME	0	6	9	14	7	6	15	15	0	0	0	0	0	0	0	0

Table 4.2. B cell effectors have cell-mediated natural cytotoxic activity (expt 2)

effector	target	LPS-treated effectors								conditioned media				conditioned media made in the presence of 10ME			
		1:4	1:1	4:1	16:1	1:4	1:1	4:1	16:1	0.8%	3.1%	13%	50%	0.8%	3.1%	13%	50%
L10A2.J	B/CN	0	0	6	3	0	0	4	11	0	0	1	0	0	1	0	0
L10A2.J	10ME	15	24	37	41	31	35	56	52	1	1	0	12	0	0	0	0
18-48	10ME	6	18	31	37	13	30	53	48	0	0	0	27	0	0	0	0
ABPL2.2	10ME	8	12	18	17	26	24	21	11	5	8	4	10	0	0	0	0
ABPL109.1	10ME	7	9	20	14	17	21	28	7	0	0	6	7	0	0	0	0
WR13.2	10ME	0	22	38	21	15	27	48	55	0	0	0	0	0	0	0	0
WR313.4	10ME	0	0	0	11	0	3	1	11	0	0	0	0	0	0	0	0

Table 4.3. B cell effectors have cell-mediated natural cytotoxic activity (expt 3)

effector	target	LPS-treated effectors								conditioned media				conditioned media made in the presence of 10ME				
		1:4	1:1	4:1	16:1	1:4	1:1	4:1	16:1	0.8%	3.1%	13%	50%	0.8%	3.1%	13%	50%	
L10A2.J	B/CN	0	1	4	0	1	7	9	6	0	0	0	0	0	0	0	0	0
L10A2.J	10ME	11	29	47	32	17	33	50	41	0	0	0	6	0	0	1	3	
2PK3	10ME	14	33	70	86	12	30	60	63	0	0	0	6	0	1	3	4	
RAW8.1	10ME	7	17	27	28	9	18	24	26	0	0	0	6	0	1	0	0	
RAW108	10ME	0	6	16	20	6	11	20	23	0	0	0	0	2	0	0	0	
RAW109	10ME	19	26	22	17	18	22	18	7	4	2	3	9	1	0	0	3	
RAW117.4	10ME	31	37	35	29	27	29	29	19	0	0	1	5	2	0	1	4	
RAW253.1	10ME	3	4	6	6	0	0	0	0	0	0	1	2	1	0	0	0	
RAW311.2	10ME	0	1	0	0	0	0	2	1	3	3	2	2	1	2	3	5	

Table 4.4. Summary of the phenotypes of B cell lines

cell line	method of transform.	B type ^a	cell type ^a	NC activity ^b	soluble lytic activity ^c
ABPL 2.2	AbMuLV	preB	PL ^d	+	±
ABPL 4.3	AbMuLV	preB	PL	+++	-
ABPL 109.1	AbMuLV	preB	PL	+	-
L10A2.J	spont.	B	lymphoma	++	-
RAW 8.1	AbMuLV	preB		++	-
RAW 108	AbMuLV	preB	lymphoma	+	-
RAW 109	AbMuLV	preB	lymphoma	++	-
RAW 112.2	AbMuLV	preB	lymphoma	+	-
RAW 117.4	AbMuLV	preB	lymphoma	++	-
RAW 253.1	AbMuLV	preB		-	-
RAW 306.3	AbMuLV	preB	lymphoma	+	-
RAW 309F.3	AbMuLV	preB	PL	±	-
RAW 311.2	ABMuLV	B	myeloma	-	-
WEHI 231.1	mineral oil	B	lymphoma	+	-
WR13.2	AbMuLV	preB		++	-
WR313.4	AbMuLV	preB		±	-
18-48	AbMuLV	preB	lymphoma	++	+
2PK-3	mineral oil	early		+++	-

^a Raschke, 1980; W.C. Raschke, The Salk Institute, personal communication

^b relative to L10A2.J (L10A2.J = ++)

^c relative to cell-associated activity

^d PL= plasmacytoid lymphoma

Lipopolysaccharide [LPS], *E. coli* endotoxin, has been shown to activate macrophages and induce the secretion of TNF (Spengler et al, 1993; Urban et al, 1986). LPS is also known to activate B cells as a substitute for antigen activation. The effect of LPS on the B cell lines was tested to determine whether these effects can be observed for NC activity (complete data are described on Tables 4.1, 4.2, and 4.3). LPS did not significantly enhance the killing of our B cell lines. Thus, NC activity is truly a "natural" response that is likely to be constitutively expressed.

Several groups have reported that TNF or LT is secreted by B cells (Ware et al, 1992; Laskov et al, 1990; Yamanaka and Karpas, 1989). If this is true of the NC activity that we are observing for L10A2.J, then this would suggest that cell-to-cell contact is unnecessary for NC-mediated killing. To assess this, we compared the amount of specific lysis achieved by B cell supernatants on 10ME targets. Accordingly, if the lysis was due to release of TNF, then we would expect to detect lytic activity in these supernatants. Supernatants (conditioned media) were taken from 18 hr cultures of B cell lines or from 18 hr co-cultures of various B cell lines and 10ME. The co-culture supernatants were produced at a B cell:10ME ratio of 4:1 since we observe significant activity at this E:T. The majority of B cell supernatants that had been prepared in the presence or absence of 10ME stimulator cells showed either no killing or very weak killing, i.e., less than 64-fold weaker than the cell-associated killing (Fig 4.1B and Tables 4.1, 4.2, and 4.3). Supernatants of co-cultures of L10A2.J, RAW 8.1 and WEHI 231 with 10ME at E:T of 1:1 and 16:1 resulted in comparably low levels of soluble cytolytic factors. It is conceivable that TNF was degraded by the B cells in the presence of 10ME or adsorbed by the targets before it could be assayed. However, this possibility appears unlikely since we have shown that TNF added

to NC assays can be recovered after 18 hr co-cultures comparable to those reported here (Patek and Lin, 1989). Thus, L10A2.J and other B cells use a cell-associated, rather than a secreted lytic molecule. Of course, this experiment does not preclude the possibility that B cells could be killing targets by secreting a lytic molecule locally during cell-to-cell contact.

This evidence suggests that the mechanism of killing is via cell-cell contact and does not result from a secreted factor. Furthermore, LPS treatment does not increase the lytic activity of supernatants. Because the B cell lines tested were of diverse stages of development and transformed by several methods, NC activity may be associated with various B cells independent of either their stage of differentiation or their ability to secrete immunoglobulin.

b. B cell effectors are NC effectors

An important characteristic to verify that other B cell lines are NC effectors is the ability to efficiently lyse NC targets, but not NK targets, in an 18-hour assay and not lyse any targets in a six hour assay. For the experiment, RAW 8.1, an Abelson-murine leukemia virus- [AbMuLV] transformed pre B cell line with NC-like activity was utilized. Figure 4.2 show that while spleen cells manifest efficient killing of both NC and NK targets, L10A2.J and RAW 8.1 only killed 10ME efficiently.

TNF has been demonstrated to play a major role in NC killing of target cells (Ortaldo et al, 1986; Patek et al, 1987; Vanderslice and Collins, 1991). Thus, if B cell lines are mediating NC activity it is expected that they will utilize TNF. The addition of anti-TNF antibody to a cytolytic assay greatly reduces the killing of targets by NC effectors (Patek et al, 1987; Ortaldo et al, 1986).

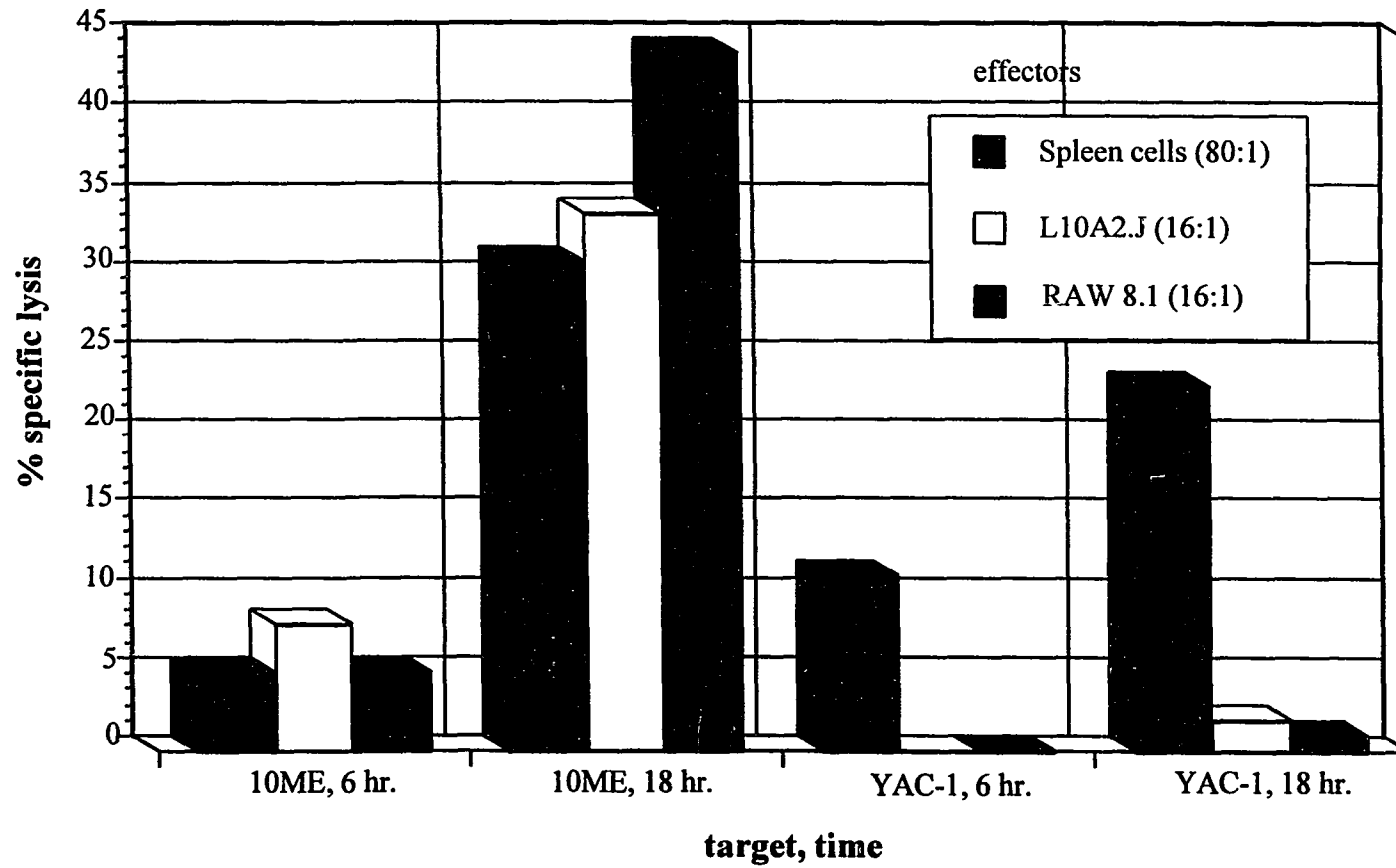


Figure 4.2. L10A2.J and RAW8.1 kill NK targets and not NK targets.

Similarly, anti- TNF antibodies inhibit killing of 10ME by L10A2.J, RAW 8.1, and WEHI 231.1 as well as by splenic NC and TNF (Fig. 4.3).

We were interested in whether NC activity was also inherent in normal B cell populations. To address this possibility, lymphocytes were separated from spleen cells by Ficoll-Paque gradient separation, and then treated with antibody and complement to eliminate either B or T lymphocytes (Fig. 4.4). Antibody against Thy-1, a murine T cell marker, or murine immunoglobulin, failed to reduce the lytic activity against 10ME to a level below control treated populations. Thus, both T lymphocytes and B lymphocytes have NC activity. Our results confirm those of earlier experiments (Paige et al, 1978) and are confirmed by more recent experiments (Lopez-Cepero, 1994) showing that B cell populations can mediate killing of certain target cells. B cells purified from spleen cell populations also have been shown to have cytotoxic activity against mouse hepatitis virus-infected cells (Nishioka and Welsh, 1993).

c. Conclusions

Recently certain cultured pre-B and B cell supernatants have been found to contain TNF and LT (Yamanaka and Karpas, 1989; Laskov et al, 1990; Ware et al, 1992; Lopez-Cepero, 1994). The production of these cytokines appears to be limited to specific stages of B cell differentiation. Thus, it is possible that L10A2.J, or other transformed B cell lines, are secreting TNF or LT either constitutively or upon stimulation by target cells. To test these possibilities, several pre-B and B cell lines were tested for their ability to lyse NC-sensitive target cells and for the ability to secrete TNF or other cytolytic factors. Our findings show that several pre-B and B cell lines express NC-like activity even

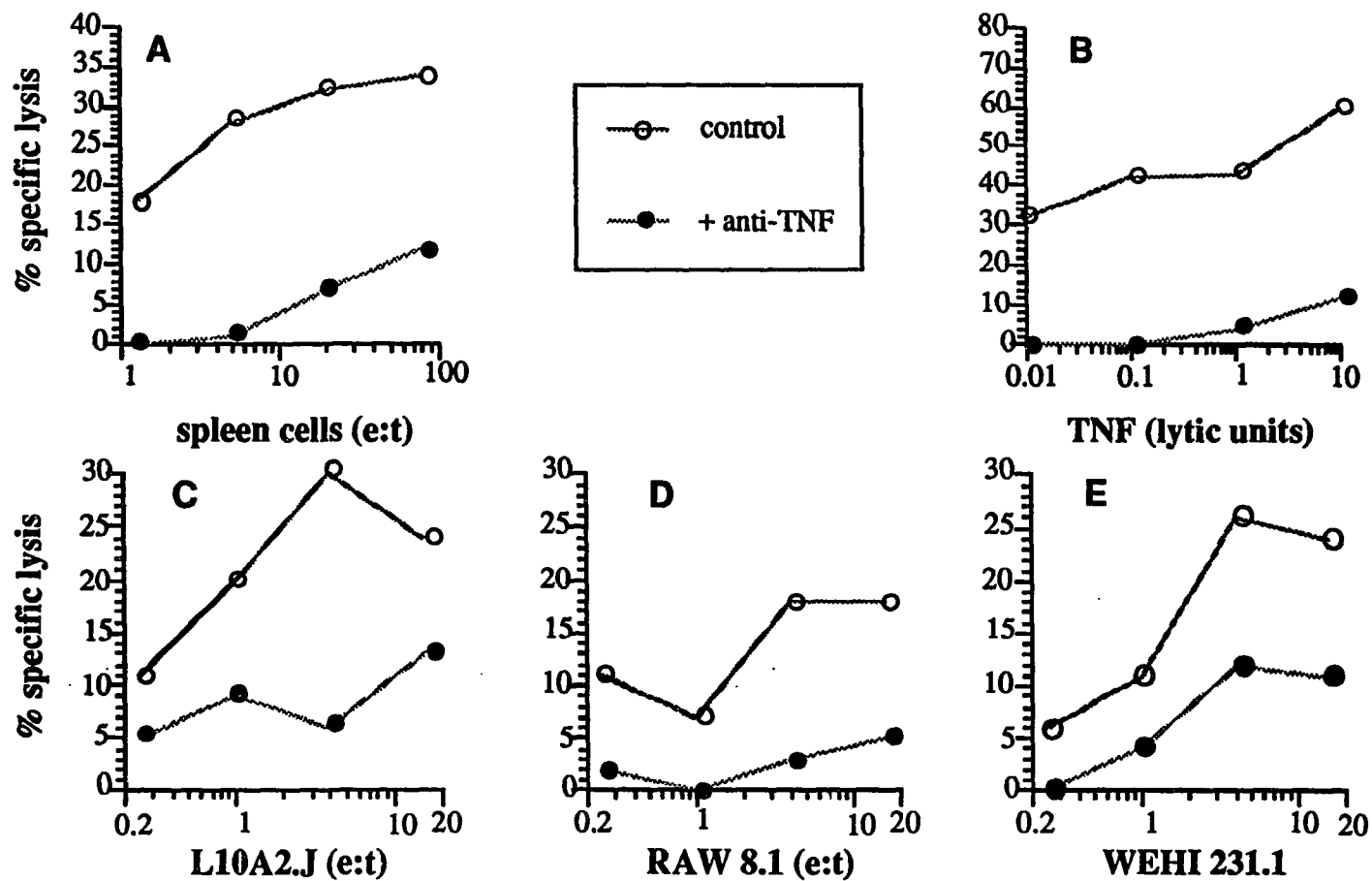


Figure 4.3. B cell line natural cytotoxic activity against 10ME targets is inhibited by antibody to TNF.

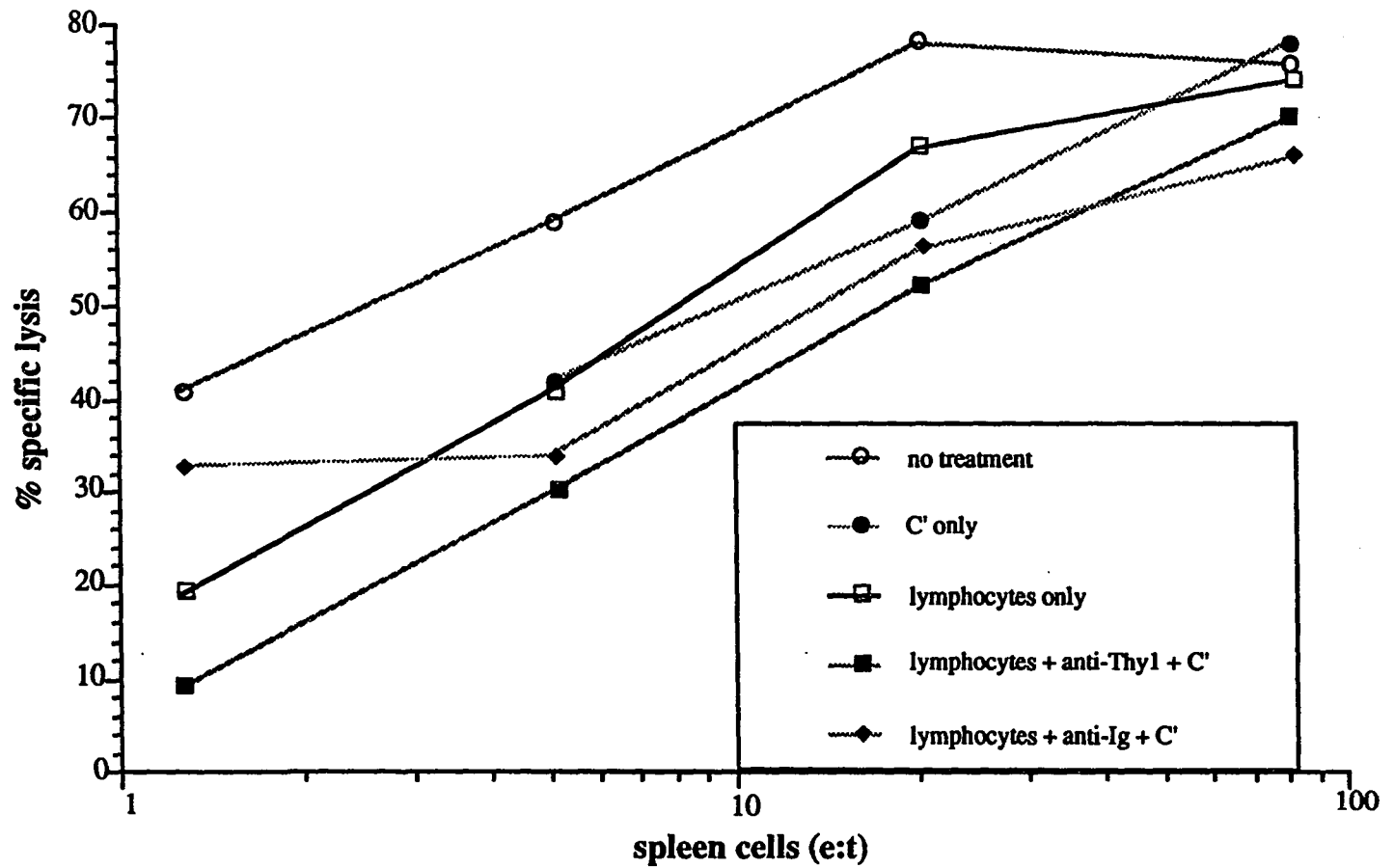


Figure 4.4 Antibody with complement selection against T cells or B cells does not reduce NC activity against 10ME targets.

though they usually secrete no cytotoxic factors (Table 4.4). It is conceivable that TNF is secreted then degraded in the co-cultures so that we would fail to detect it in our assays. However, 18 hr. co-cultures of TNF with targets and effectors do not degrade the activity of the TNF in the supernatants (Patek and Lin, 1989). Thus, it is unlikely that significant quantities of TNF are secreted. It is also noteworthy that two of the B cell lines, 18-48 and ABPL2.2, secrete a small amount of a cytolytic factor; however, the secreted cytolytic activity from these cell lines can account for only one-fourth of the cell lines lytic potential. Accordingly, it is presumed that these cells also mediate cytolysis via effector-target contact.

These data suggest that the NC-like activity first described for L10A2.J is a common phenotype of transformed B cells and that there is no apparent correlation between the differentiation stage of the B cell and presence of the NC-like activity. Since the cytolytic activities of L10A2.J, RAW 8.1, and WEHI 321 are blocked by anti-TNF antibody (Fig. 4.3), it appears that certain B cells mediate an immunoglobulin-independent, cytolytic activity that utilizes TNF as its effector. This argument is strengthened by the fact that normal B cell populations as well as these transformed cell lines have NC activity (Fig. 4.4; Lopez-Cepero et al, 1994). Furthermore, these results are consistent with the hypothesis that NC activity is mediated primarily by effector-target contact and not secreted TNF as some investigators have claimed (Ortaldo, 1986). Accordingly, these cell lines, along with cloned thymic lymphomas with NC-like activity (Lattime and Stutman, 1991), may prove critical for analysis of target recognition and lytic mechanisms of NC effectors.

Chapter 5. Killing of targets by NC effectors requires effector-target binding

The second objective of this project is to determine the importance of binding of NC effectors to targets. In Chapters 5 through 8, we show that binding is important for NC activity. Using L10A2.J as a cloned NC effector, we demonstrate in this chapter that binding is important for killing of targets by NC effectors.

a. Development of the ⁵¹Cr-labeled effector binding assay

Binding by NC effectors has not been studied previously. This is because spleen cell target binding is inherently difficult to assess due to the fact that heterogenous spleen cell populations consist of macrophages, T lymphocytes, and other leukocytes as well as B cells and any or all of these may contribute to splenic NC activity (Paige et al, 1978; Figure 4.4). Likewise, while many studies have investigated the binding of TNF to target cells, we can not determine the precise role of TNF in effector-target binding since TNF is a single molecule and NC effectors contain numerous molecules on the surface, many of which may be involved in binding. Now that we have cloned NC effectors such as L10A2.J, it is feasible to study binding of effectors to targets.

In running cytotoxicity assays, we noticed that rosettes are formed by L10A2.J on target cells. When 1×10^5 10ME cells are plated on a 60 mm tissue culture dish, they generally have a rounded morphology with some cell membrane portions attached flatly to the tissue culture dish as they are adherent cells (Fig. 5.1A). L10A2.J, on the other hand is a nonadherent cell usually appearing as spheres suspended in the medium (Fig. 5.1B). When 1×10^5 L10A2.J is added to the plated 10ME cells (i.e., a 1:1 effector:target ratio), rosettes are observed (Fig. 5.1C).

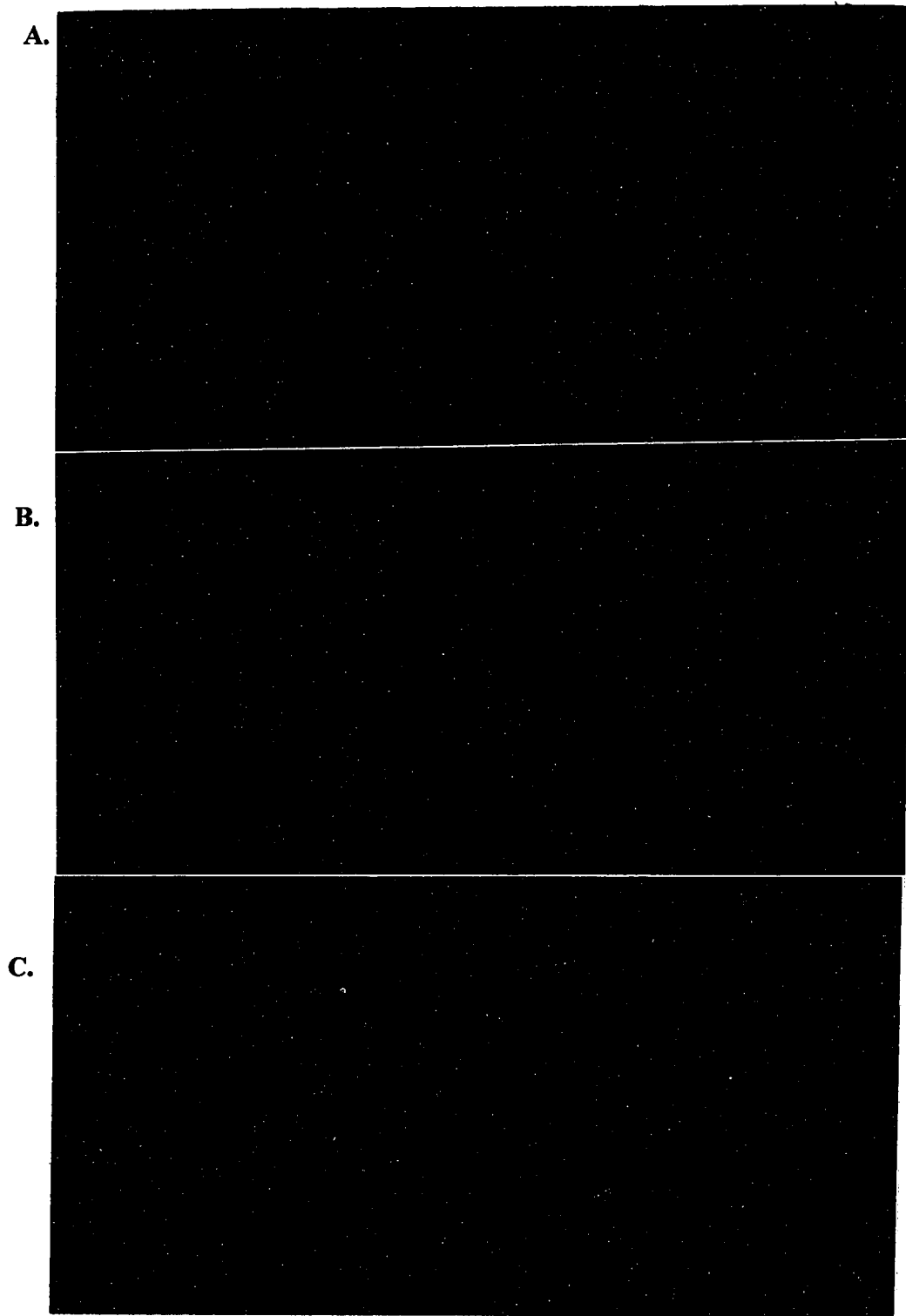


Figure 5.1. L10A2.J forms rosettes on 10ME targets. A; 10ME. B; L10A2.J. C; 10ME and L10A2.J (1:1).

We used this property of NC effectors and targets to develop a suitable assay to study binding by NC effectors to NC targets. Sodium chromate is taken up by living cells and can be used as a marker of cells. ^{51}Cr labeling is commonly used in viability or cytotoxicity tests, and also as a marker of cell migration in short term in vivo assays (Cook and Mitchell, 1989). Accordingly, a ^{51}Cr -labeled effector binding assay was developed.

To analyze the feasibility of this assay, we first tested the proportion of L10A2.J cells that remained unbound, and the proportion that become specifically bound to 10ME targets. In the first experiment, ^{51}Cr -labeled 10ME was allowed to adhere to the tissue culture dish for 30 minutes after which unlabeled L10A2.J was added and allowed to incubate while rocking for 1 hour. As shown in Figure 5.2A, the majority of 10ME was bound to the tissue culture dish (66% strongly adherent, 18% weakly adherent). 16% of 10ME had not adhered to the plate by 90 minutes (light washes). The non-adherent and weakly adherent populations may have been due to the short amount of time allowed for adherence after the labeling procedure. 10ME cells cultured overnight appear to have very few non-adherent or weakly adherent populations. In the second experiment, labeled L10A2.J was added to a tissue culture dish of unlabeled 10ME that had been grown overnight at 4:1 e:t (Fig. 5.2B, Fig. 5.2C). 44% of L10A2.J cells were unbound or weakly bound (29% in the supernatant, 15% recovered in the light washes), and 55% were strongly bound to 10ME. 1% of the labeled L10A2.J remained adhered to the tissue culture plate after hard washes. We conclude that a 55% binding of effector cells to target cells is reasonable. Because L10A2.J can form rosettes on 10ME targets (Fig. 5.1C), and because a 4:1 e:t was used, on average 2.2 L10A2.J cells bound to each 10ME target. It is possible that NC effectors do not need to bind for a long duration to initiate apoptosis in targets. Alternatively, the 44% unbound L10A2.J could be due to a normal proportion of the population that is nonbinding or due to a saturation of binding sites by

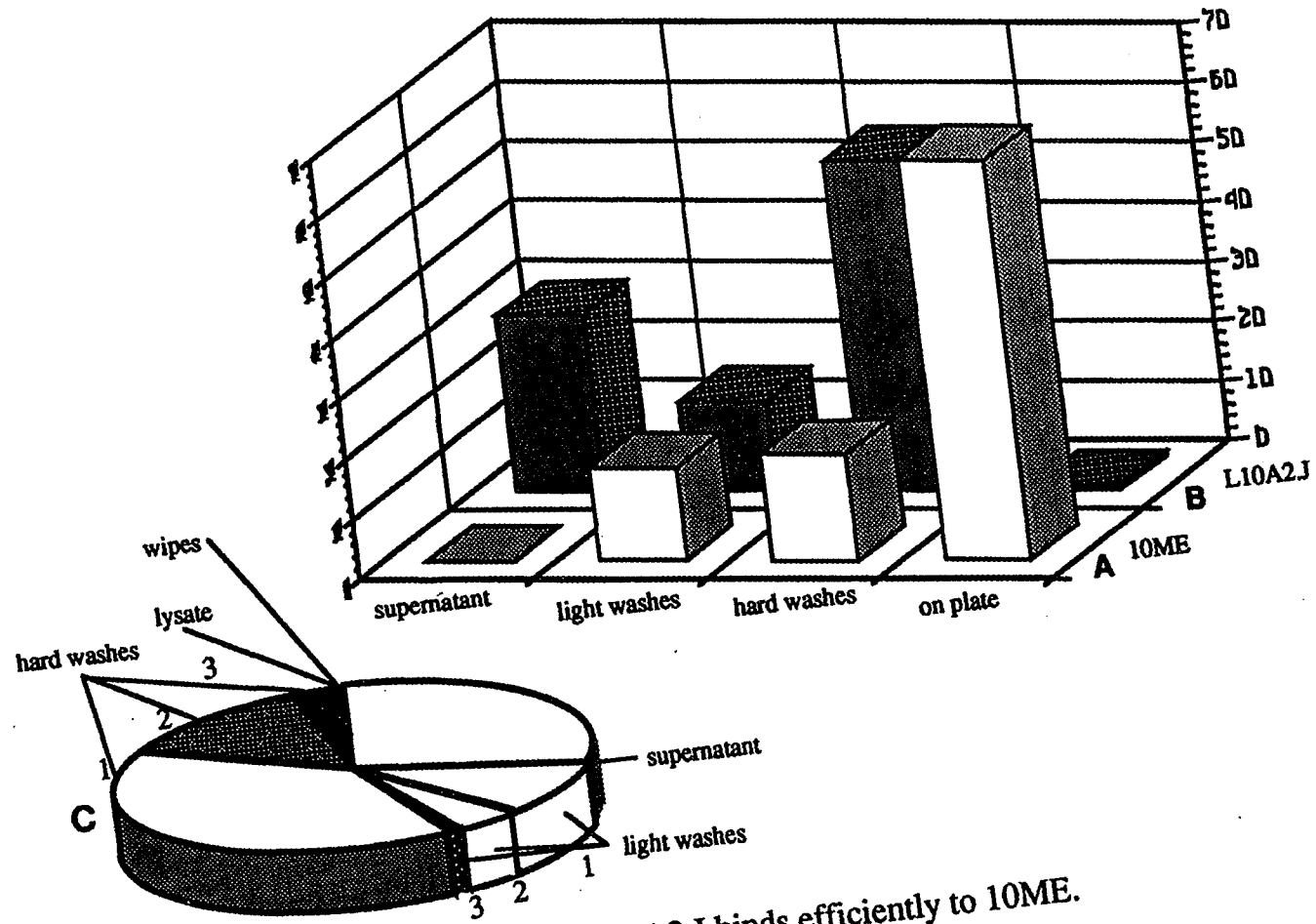
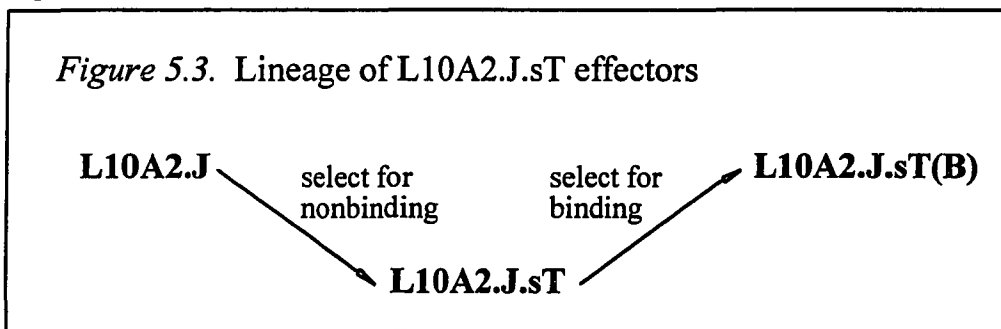


Figure 5.2. L10A2.J binds efficiently to 10ME.

bound L10A2.J. This experiment showed us that ^{51}Cr -labeled L10A2.J could be used to analyze binding of NC effectors to targets.

b. Selection of L10A2.J nonbinding variants

In order to confirm the effectiveness of the ^{51}Cr -labeled effector binding assay, we selected a population of L10A2.J for reduced binding (refer to Ch. 2 Materials and Methods; Fig 5.3). Briefly, L10A2.J was added to adherent 10ME cells and incubated with rocking for 30-60 minutes. Nonadherent L10A2.J were then removed and cultured. After six or more selection cycles, the resulting population, L10A2.J.sT was then tested for its ability to kill target cells (Fig. 5.4). L10A2.J.sT was approximately 64-fold less efficient at the killing of 10ME compared to the parent L10A2.J. The fact that L10A2.J.sT has reduced cytotoxicity also confirms the hypothesis that binding is important for killing.



A problem encountered in the selection of L10A2.J.sT is that the nonbinding phenotype is unstable. The binding phenotype is recovered within 6 days (Fig. 5.5). Through day 3 after the selection, there is a substantial difference between killing by L10A2.J.sT and parent L10A2.J at 4:1 effector:target ratios. By day 5, the difference in killing is minimal; by day 6, the difference is negligible. To address this problem, we cloned newly selected L10A2.J.sT. Of 20 clones cultured, 13 clones survived and were able to grow to a substantial number by three weeks after cloning. These clones were tested for the ability to kill 10ME (Table 5.1). Clones sT.2, sT.7, sT.8, sT.9, sT.13, and

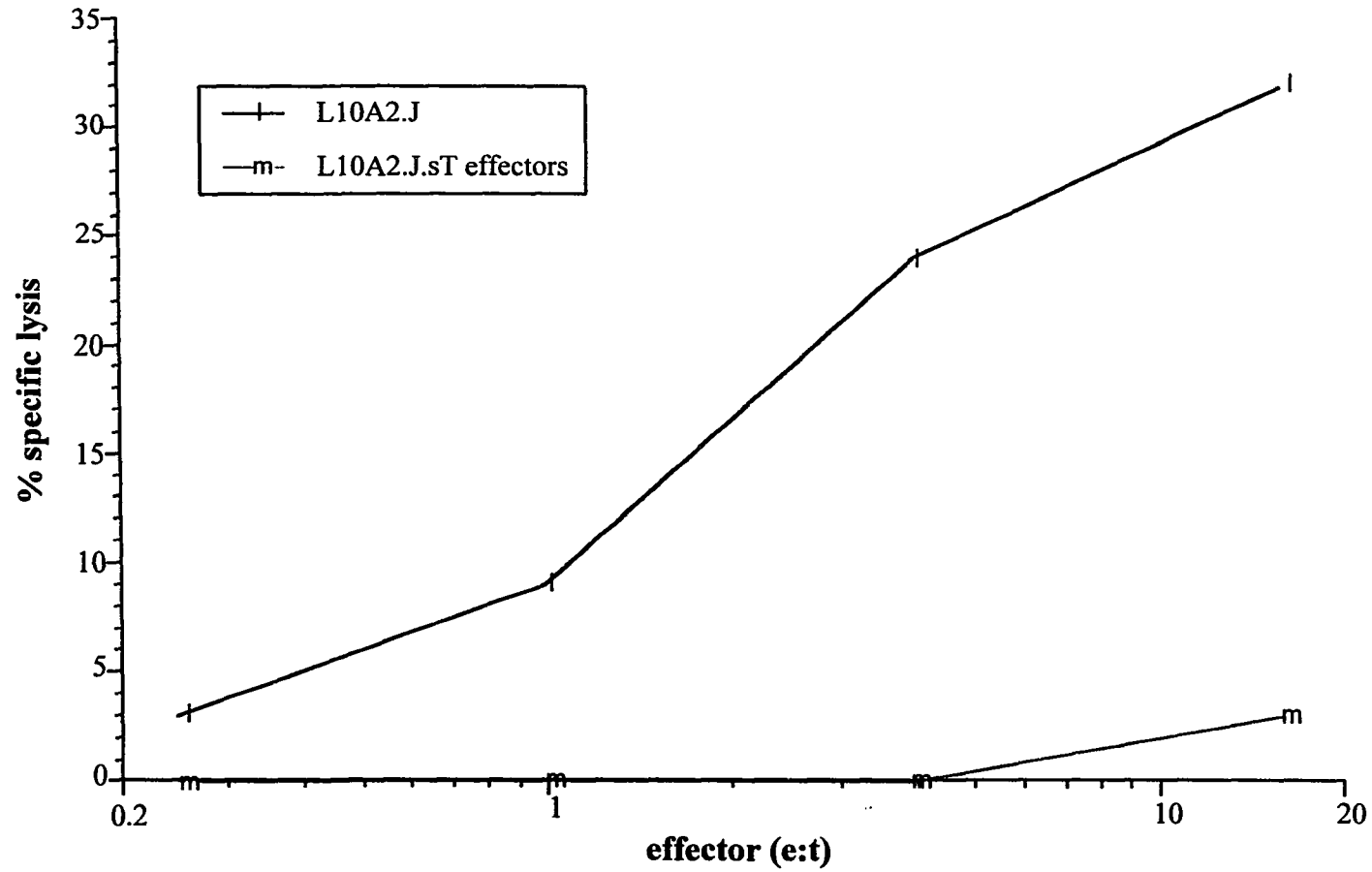


Figure 5.4. L10A2.J selected for reduced binding has reduced killing of 10ME.

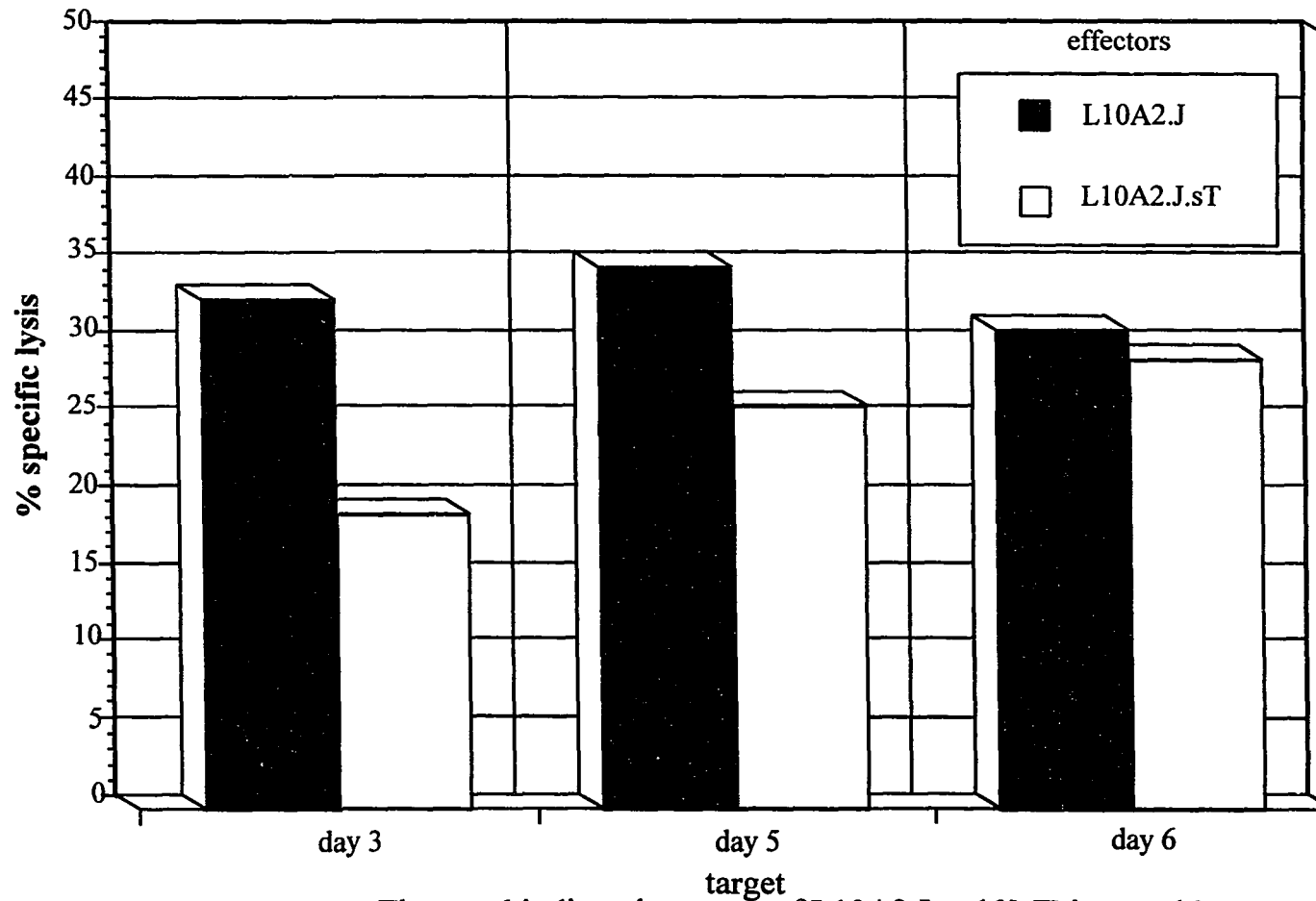


Figure 5.5. The non-binding phenotype of L10A2.J to 10ME is unstable.

Table 5.1. Cloning of non-binding L10A2.J
(expt 1 = 3 weeks after cloning)

effector	1:4	1:1	4:1	16:1
L10A2.J	5	31	56	61
sT.2	0	7	24	43
sT.5	5	23	40	21
sT.6	6	22	31	26
sT.7	3	16	35	38
sT.8	1	14	28	26
sT.9	1	11	33	39
sT.10	0	n.d.	41	43
sT.11	3	20	42	45
sT.12	7	20	39	35
sT.13	2	12	35	34
sT.16	1	13	33	25
sT.17	6	26	47	43
sT.18	4	19	42	46

sT.16 were roughly four-fold lower compared to parent L10A2.J. sT.5, sT.6, sT.10, sT.11, sT.12, sT.17, and sT.18 had slightly reduced killing compared to L10A2.J. After seven weeks, all of these cell lines were nearly the same as L10A2.J (Table 5.2). The inability to achieve a stable cloned cell line indicates that the binding phenotype is variable or regulated. Alternately, the binding property may give cells a growth advantage such that there is constant in vitro selection for that phenotype.

Because there is a correlation between nonbinding and reduced killing, we then used the L10A2.J.sT population to further assess the ^{51}Cr -labeled effector binding assay. A population, such as L10A2.J.sT, that is selected for reduced binding should also show this property in the ^{51}Cr -labeled effector binding assay we have developed. L10A2.J.sT has substantially less binding to 10ME targets than does the parent L10A2.J or L10A2.J.sT(B), a variant of L10A2.J.sT which was reselected for the ability to bind 10ME (Fig. 5.6). These results confirm that the ^{51}Cr -labeled effector binding assay offers a valid means to measure effector-target binding.

c. Conclusions

We have demonstrated that we can use the ^{51}Cr -labeled effector binding assay to study binding of NC effectors to targets. Because the population of L10A2.J selected for nonbinding, L10A2.J.sT, demonstrated reduced binding, and the population reselected for binding, L10A2.J.sT (B) demonstrated normal binding, the ^{51}Cr -labeled effector binding assay is a feasible means to assess binding of effectors and targets. Furthermore, this binding is specific for effectors and targets capable of binding each other because the small amount of nonspecific binding (1-10% in all valid assays performed) was accounted for by subtraction from both bound and total counts (refer to Ch. 2 Materials and Methods). We now have a means to analyze the binding of cell effectors to targets, thus we use the ^{51}Cr -labeled effector binding assay throughout the rest of this project.

*Table 5.2. Cloning of non-binding L10A2.J
(expt 2 = 7 weeks after cloning)*

effector	1:4	1:1	4:1	16:1
L10A2.J	27	53	65	50
sT.2	19	33	48	38
sT.5	15	30	45	37
sT.6	11	28	45	44
sT.7	16	32	49	48
sT.8	17	39	52	48
sT.9	20	40	57	47
sT.10	17	39	52	48
sT.11	28	49	63	50
sT.12	22	39	57	50
sT.13	21	40	52	50
sT.16	19	41	49	37
sT.17	21	42	57	50
sT.18	18	41	54	51

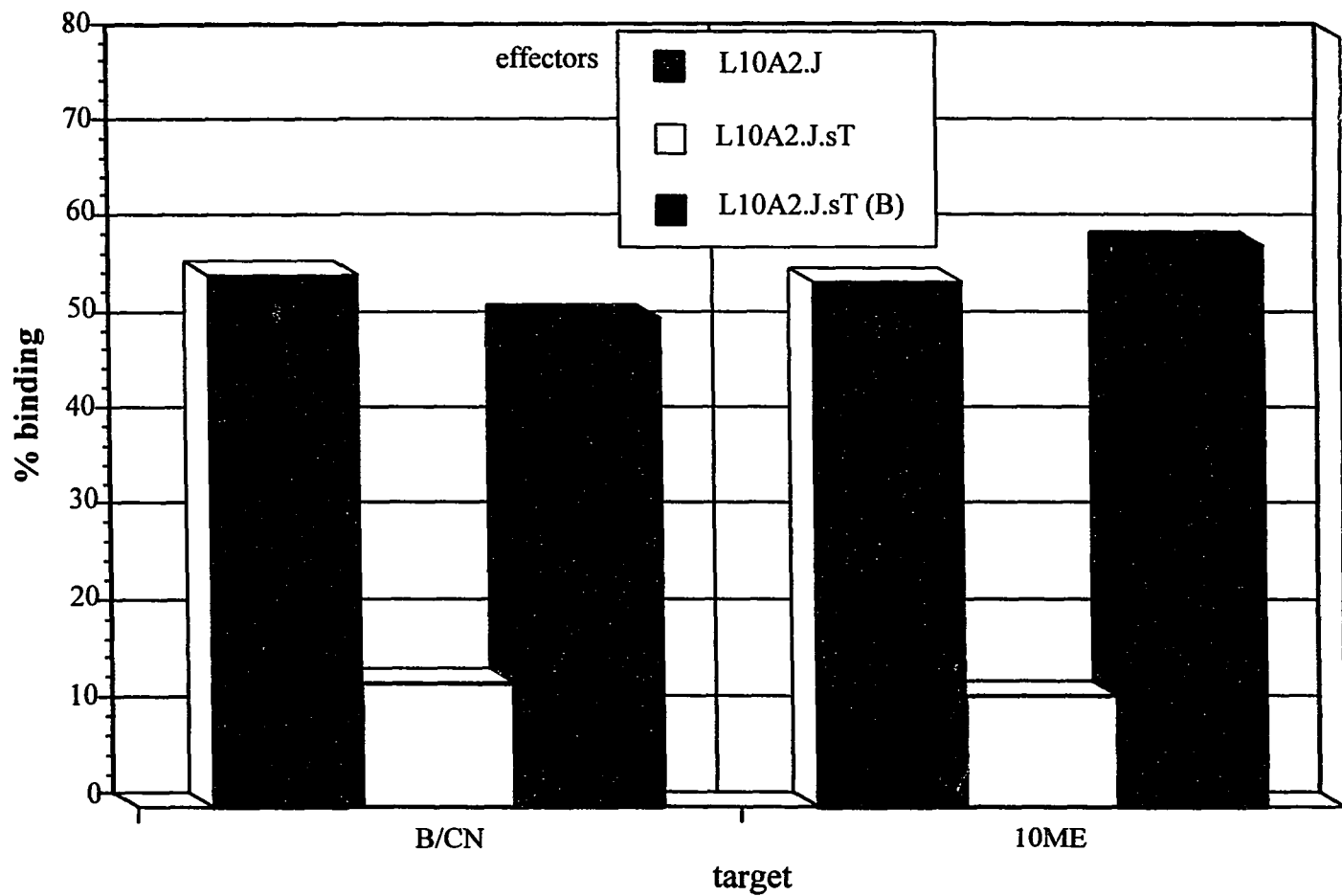


Figure 5.6. L10A2.J selected for nonbinding has reduced binding to 10ME.

In addition, we have demonstrated here that binding is important for the killing of 10ME targets by L10A2.J NC effectors. L10A2.J.sT, the variant of L10A2.J which was selected for reduced binding, is less efficient at killing. While the phenotype is transient, the reduction of killing by L10A2.J.sT is significant. In Chapter 6 we assess the significance of binding in NC-mediated cytotoxicity and immune surveillance, and in Chapters 7 and 8 we address some potential mechanisms of NC effector binding.

Chapter 6. NC activity involves recognition or binding elements as well as lytic elements

NC effectors mediate cell-associated killing of transformed target cells via cell surface TNF (refer to Ch. 3 and Ch. 4). Thus it is logical to assume that recognition and binding may play an important role in the killing of target cells by NC effectors. The fact that N-lines are resistant, I-lines are sensitive, and C-lines are resistant to NC-mediated lysis suggests that, at some level, there is a discrimination of targets. Until now, it has been uncertain as to how much of this discrimination is due to the reaction of the target cell to TNF and how much has to do with target recognition and binding. Normal cells are probably resistant to TNF due to the initiation of a transcription and protein synthesis-dependent protective signal as well as a lytic signal by TNF (Collins et al, 1981; Beyaert and Fiers, 1994). For some reason, the transformed target cells apparently lose this protection, or the lytic signal is turned on to a high enough level that the protection does not abrogate the lytic signal. It is also possible that secondary signals or co-stimulatory signals come into play that allow NC effectors to be more efficient than soluble TNF at killing transformed targets.

Given that binding of NC effectors to targets occurs, we can hypothesize that this binding allows for secondary signals and "cross-talk" between effectors and targets. Perhaps a recognition step is necessary, in which transformed targets are more readily recognized by NC effectors than are normal targets. It is possible that transformed cells express higher levels of a binding determinant. Finally, TNF-selected cells differ from NC-selected cells (both spleen cell- and L10A2.J-selected) in that the TNF-selected I-cell lines are nontumorigenic in normal mice while NC-selected I-cell lines are tumorigenic (i.e., they become C-lines). Perhaps recognition can elucidate the differences between NC effectors and TNF. Alternatively, binding may have no role in recognition at all and

merely aids in efficiently keeping TNF and the target cell in close proximity, perhaps also prohibiting TNF/TNFR recycling.

a. C-lines have reduced binding

In the N-I-C model, N-lines are normal, nontumorigenic cell lines, I-lines are transformed cell lines that are tumorigenic only in immune deficient mice, and C-lines are transformed cell lines that are tumorigenic in both immune deficient and normal mice (Patek et al, 1978; Collins et al, 1981). B/CN, the N-line, is resistant to NC-mediated lysis (by spleen cells or L10A2.J) and does not form tumors in mice. Theoretically, this is due to the fact that the immune system should not attack normal cells unless undergoing an autoimmune reaction. 10ME, the I-line is sensitive to NC-mediated lysis and forms tumors in ATXFL and athymic nude mice, but not in normal mice. Presumably, the immune system recognizes and kills these abnormal cells. L88, the C-line is resistant to NC-mediated lysis and forms tumors in normal mice. It is conceivable that L88, which was selected for its ability to grow as a tumor, is not only NC-resistant, but has gained some way to escape the recognition and killing by NC effectors. Because L88 is also resistant to TNF-mediated killing, it is possible that TNF-resistance is one means by which transformed cells can escape surveillance by NC effectors. However, it is possible that there is more than one escape mechanism operating at a time in C-lines, because each escape mechanism is selectively favorable to the tumor (i.e., multiple escape mechanisms are highly favorable).

Indeed, there is significant evidence that the transition from I-line to C-line is mediated, in part, by a loss of NC recognition of the target cells. We have found that B/CN and 10ME bind L10A2.J, whereas L88 shows reduced binding (Fig. 6.1). These data suggest that both B/CN and 10ME efficiently interact with NC effectors and that the.

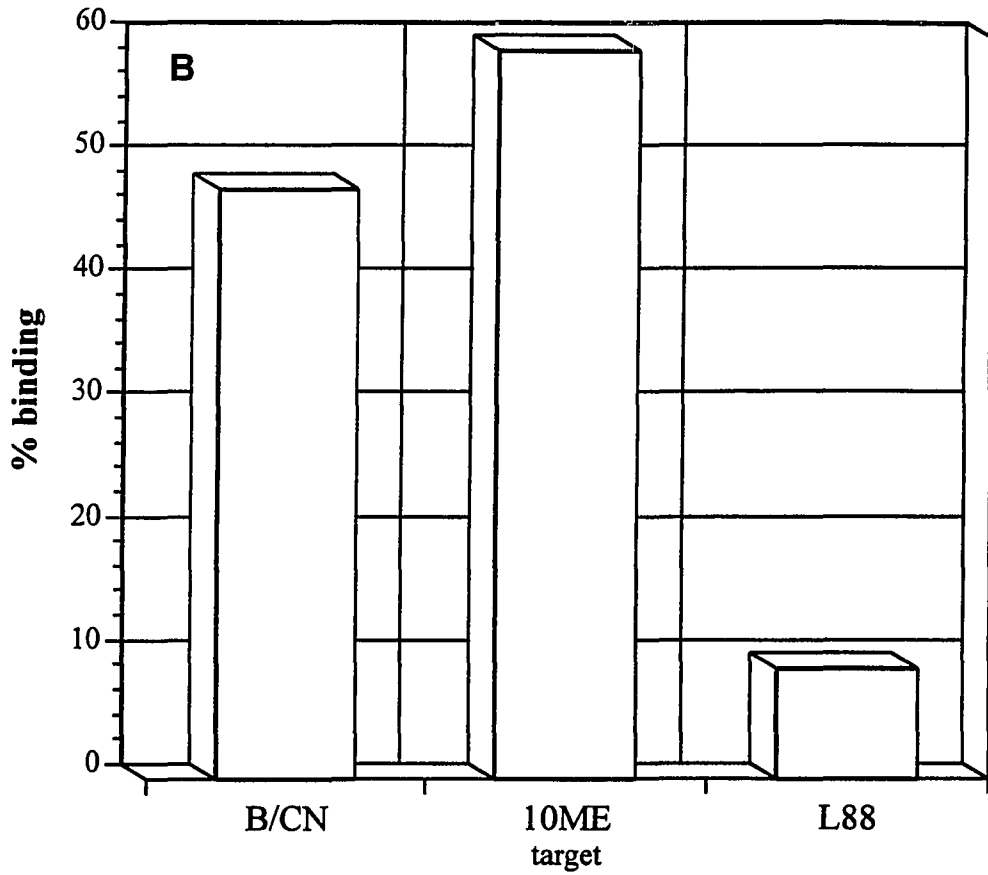
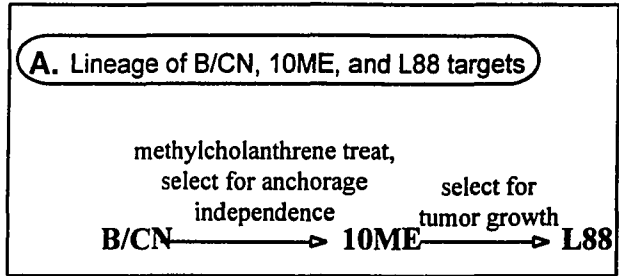


Figure 6.1. L10A2.J natural cytotoxic effectors do not bind L88.

discrimination between these targets takes place at the level of the TNF protective signal. On the other hand, L88 has reduced its binding to NC effectors. This could be explained by a reduction of recognition determinants on the surface of C-lines. It seems that at least two means of escape from NC-mediated immune surveillance are found in L88, reduced recognition and post-recognition resistance to the TNF-initiated lytic signal. It is also possible that binding is not due to constitutively expressed recognition determinants, but is due to the induction or activation of binding molecules on the target or effector after the initial TNF signal is delivered.

10METO.4.4S.3 is a variant of 10ME that was selected for resistance, *in vitro*, to spleen cell-mediated killing (i.e., NC activity) (Patek et al, 1986). This cell line is resistant to NC-mediated killing (spleen cells or L10A2.J) and forms tumors in normal mice. 10METO.4.5T.1 is a variant of 10ME that was selected for resistance, *in vitro*, to TNF (Patek and Lin, 1991). This cell line is resistant to NC-mediated killing and does not form tumors in normal mice. 10METO.4.5T.1 is efficiently bound by L10A2.J whereas 10METO.4.4S.3 is not (Fig. 6.2). As with L88, L10A2.J binding correlates with tumorigenicity. Again, this indicates that escape from TNF lytic activity is insufficient for the I-lines to transition and that they must gain TNF resistance and an extra means of escape from immune surveillance by NC effectors to become C lines (i.e., they must also reduce NC binding). However, as mentioned above, it is unclear whether this indicates that there is a reduction of constitutively expressed recognition determinants, or a reduction in signals necessary for production of inducible binding elements.

Finally, we tested the cell lines that were selected for resistance to L10A2.J (Ch. 3). Cells that were selected for resistance to L10A2.J, such as 10ME.sL.2, 10ME.sL.6, and 10ME.sL.17 are NC-resistant (spleen cells and L10A2.J) and tumorigenic in normal mice, but remained somewhat sensitive to TNF. Cells that went through the selection procedure but remained NC-sensitive, such as 10ME.sL.10 and 10ME.sL.11, are only

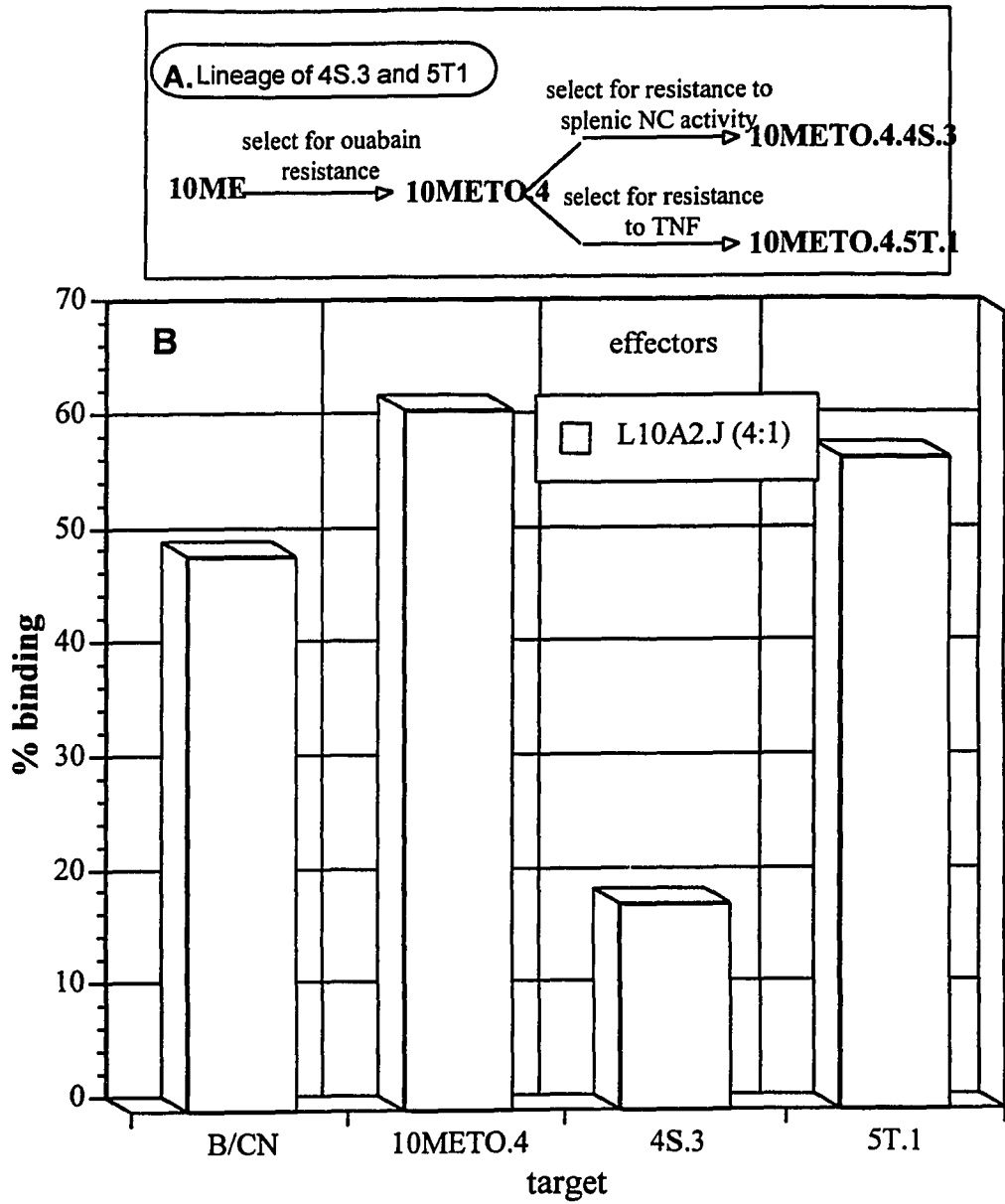


Figure 6.2. 10METO.4.4S.3 is not efficiently bound by L10A2.J

slightly less TNF-sensitive than 10ME and nontumorigenic in normal mice. The NC-sensitive cell lines (10ME.sL.10 and 10ME.sL.11) were efficiently bound by L10A2.J, whereas the NC-resistant cell lines (10ME.sL.2, 10ME.sL.6, and 10ME.sL.17) were not efficiently bound by L10A2.J (Fig. 6.3). A causal relationship between loss of L10A2.J-binding and tumorigenicity in normal mice is suggested. Again, the data show that resistance to soluble TNF is insufficient for the C phenotype (although, perhaps necessary).

b. Conclusions

Effector-to-target binding could be important to NC activity in two ways. First, binding could be due to recognition determinants which not only cause effector target adherence, but may initiate the lytic signal and, possibly, a protective signal in NC-resistant cells. Second, binding determinants could facilitate cell-to-cell adhesion and serve primarily to keep effectors and targets in close proximity so that signals can be sent.

Here we have demonstrated that binding of effectors to targets is reduced when I-line targets escape either immune surveillance *in vivo* or NC activity *in vitro*. Also, they become tumorigenic for normal mice (i.e., C-lines (Table 6.1)). This holds true for targets that are selected *in vitro* for resistance to NC effectors (spleen cells or L10A2.J) such as 10METO.4.4S.3 and 10ME.sL.6, and targets that are selected *in vivo* for tumorigenesis such as L88. Nontumorigenic transformed cell lines such as 10ME and 10METO.4.5T.1, and normal cell lines such as B/CN efficiently bind NC effectors and all of these fail to form tumors in normal syngeneic mice. Whether the function of binding is recognition of transformed targets (i.e., NC effectors are specifically targeted for the recognition determinants) or cell-to-cell adhesion (i.e., NC effectors bind to all cells except C-lines) is unclear at this time. In either case, it is important to acknowledge that binding plays a role in NC activity and appears to be a key step in preventing I-line growth *in vivo*.

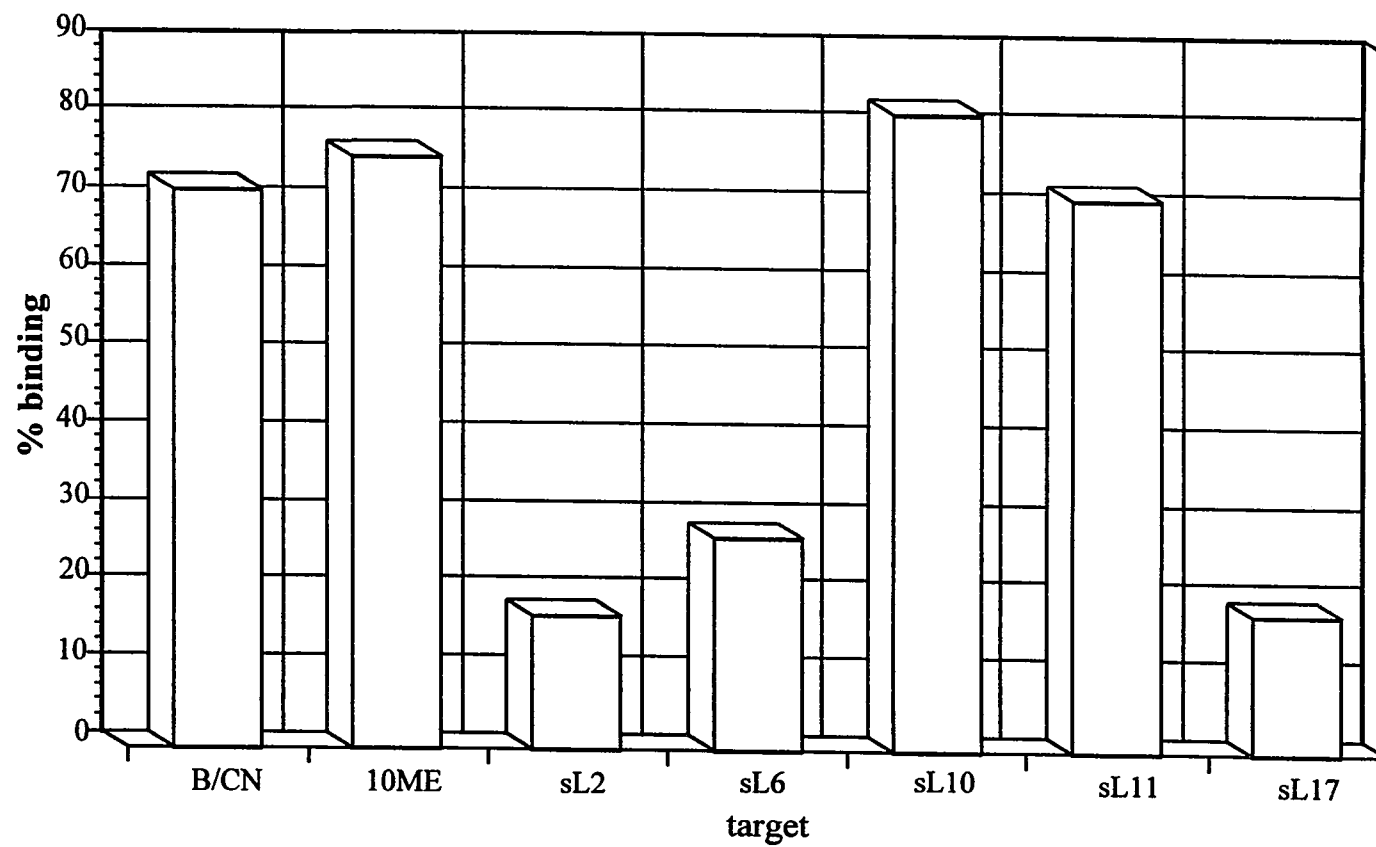


Figure 6.3. 10ME selected for resistance to L10A2.J have reduced binding to L10A2.J.

TABLE 6.1. Summary of the phenotypes of L10A2.J-selected variants of 10ME and other B/CN-derived cell lines

target cell line	parent line	selection	sensitivity to			binds NC effectors	tumors in normal mice
			s.c.	L10A2.J	TNF		
B/CN	(primary)		r ^a	r	r ^b	+	- ^c
10ME	B/CN	anc. indep [†]	s ^a	s	s ^b	+	- ^{c*}
L88	10ME	tumor	r ^a	r	r ^b	-	+ ^c
10METO.4	10ME	ouabain	s ^d	s	s ^d	+	- ^{d*}
4S.3.3	10METO.4	spleen cells	r ^d	r	r ^d	-	+ ^d
5T.1	10METO.4	TNF	r ^e	r	r ^e	+	- ^e
sL.2	10ME	L10A2.J	r	r	s	-	+
sL.3	10ME	L10A2.J	i	i	s	n.d.	n.d.
sL.5	10ME	L10A2.J	i	i	s	n.d.	n.d.
sL.6	10ME	L10A2.J	r	r	i	-	+
sL.10	10ME	L10A2.J	i	i	s	+	-
sL.11	10ME	L10A2.J	i	i	s	+	-
sL.13	10ME	L10A2.J	i	i	s	n.d.	-
sL.14	10ME	L10A2.J	i	i	s	n.d.	n.d.
sL.17	10ME	L10A2.J	r	r	i	-	+

^a Collins et al, 1981

^b Patek et al, 1987

^c Lin et al, 1985

[†] anchorage independence

^d Patek et al, 1986

^e Patek and Lin, 1991

* is tumorigenic in immunodeficient mice

Chapter 7. The role of TNF in recognition and binding of target cells by NC effectors

Tumor necrosis factor is a highly pleiotropic cytokine. The first characterization of TNF was as a factor which caused the necrosis of tumors (Carswell et al, 1975). Since then, numerous systemic, cellular, and molecular effects have been attributed to TNF. The range of cellular effects that are elicited by TNF include apoptotic and necrotic killing, cell proliferation, cell activation, and the inflammatory response (Beyaert and Fiers, 1994; Vassali, 1992; Vilcek and Lee, 1991; Fiers, 1991).

With regards to oncology, the most important of these effects is the induction of apoptotic lysis. Many transformed cells are killed by TNF-induced apoptosis, and nearly all cells can be killed by TNF-induced apoptosis in the presence of a secondary signal or a metabolic inhibitor (Beyaert et al, 1994; Wong et al, 1992; Higuchi and Aggarwal, 1994a). The apoptotic signal is exerted via the p55 TNFR (Tartaglia et al, 1991). Clustering of the TNFR by trimeric TNF initiates at least two signal mechanisms. The lytic signal appears to involve a G protein and phospholipase C signal followed by increase in inositol triphosphate and subsequent increase in intracellular calcium ion concentration. The intracellular calcium affects oxidative metabolism in the mitochondria which produce superoxide radicals. Superoxide can cause oxidative damage to a variety of molecules including DNA in the nucleus. An increase in intracellular calcium also causes an increase in nuclear calcium. Calcium-dependent endonucleases become activated which can also contribute to the degradation of DNA.

In most resistant cells, a second signal is activated which protects the cell against the lytic signal. An unknown protein kinase probably initiates this signal and causes the cleavage of NF- κ B. The resulting transcription factor can induce the expression of

several protective genes which include manganous superoxide dismutase, plasminogen activator inhibitor type-2, and the A20 zinc-finger protein (Smith et al, 1994).

a. TNF is important in the NC-mediated cytotoxicity

TNF is important in NC-mediated cytotoxicity (Patek et al, 1987; Ortaldo et al, 1986). Both NC effectors and TNF kill a similar range of target cells and cytotoxicity by these effectors exhibit similar kinetics with a 4-6 hour lag before lysis begins. Antibody against TNF also inhibits the killing of 10ME cells by splenic or L10A2.J NC effectors (Fig. 7.1A, 7.1B, respectively). However, while TNF-mediated killing (Fig. 7.1C) is completely inhibited by anti-TNF antibody, neither splenic nor L10A2.J NC-mediated killing is totally abrogated by the antibody (Fig. 7.1A, 7.1B, respectively). Three nonexclusive mechanisms could account for this discrepancy. First, NC-mediated killing requires cell-to-cell contact which could make killing via TNF more efficient and more difficult to inhibit with antibody. This is reasonable to expect in view of the fact that it is possible that in the localized space of the cell-to-cell contact, the concentration and binding affinity of mTNF to TNFR could overcome the binding of anti-TNF to TNF. Next, a secondary lytic mechanism may be present that is separate from TNF. Other lytic molecules have been described on B cells (Ni and Karpas, 1992). Finally, the antibody used may be cross-reactive to LT. LT has been found on B cells (Ware et al, 1992; Laskov et al, 1990). The antibody used here was made against mouse TNF but it is uncertain whether it cross-reacts with LT (Genzyme, IP-400). Jayaraman et al (1990) have provided some evidence that the Genzyme anti-TNF antibody may not cross react with LT, but other sources have indicated that cross reaction does occur (Genzyme Corp., Cambridge, personal communication). Thus this experiment can not definitively assign the activity to TNF or LT. Other experiments (Vanderslice and Collins, 1991) show

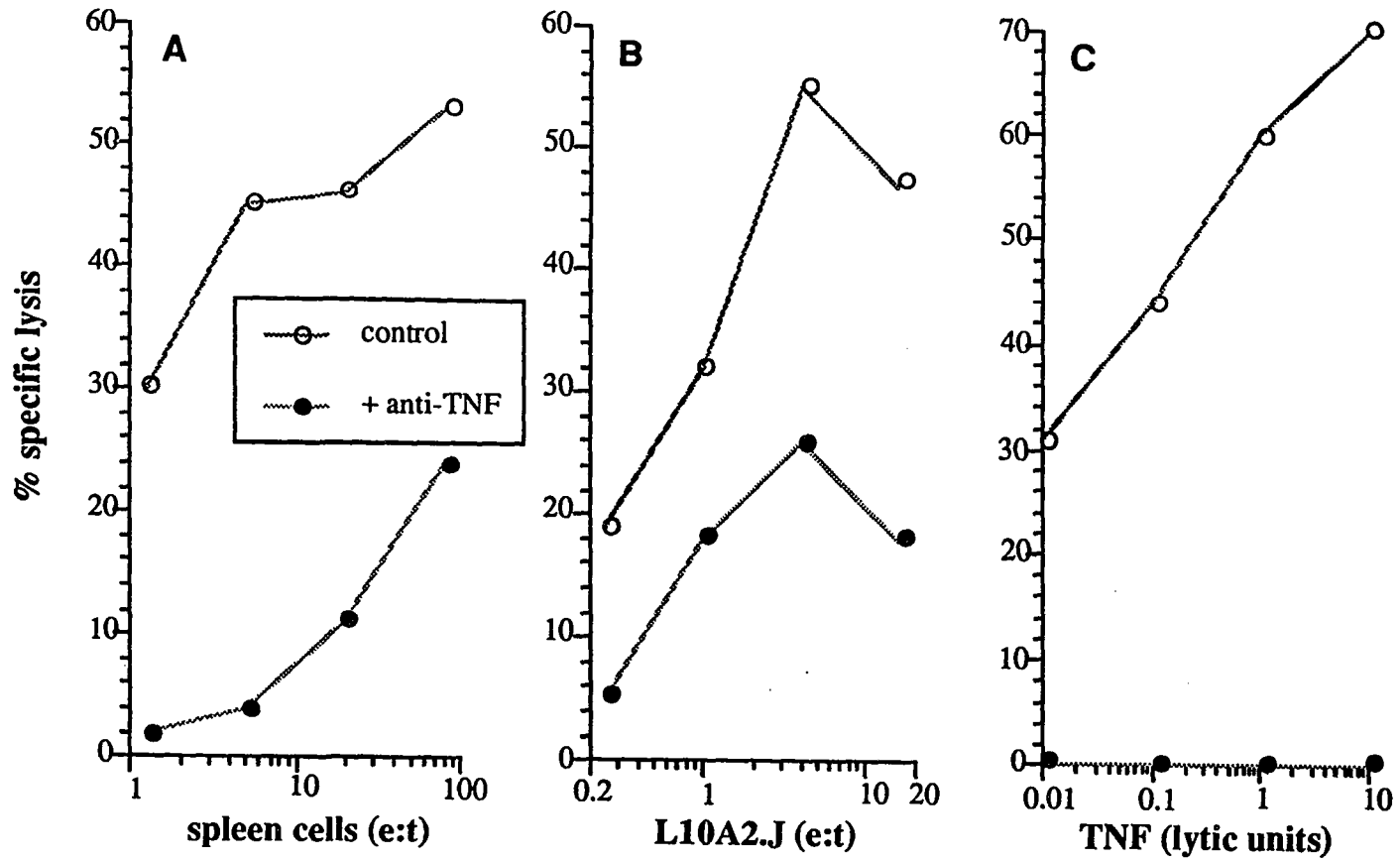


Figure 7.1. Antibodies against TNF inhibits splenic and L10A2.J natural cytotoxicity against 10ME.

that L10A2.J expresses the membrane form of TNF and attributes its lytic activity to this molecule. However, it remains possible that LT may be involved in NC activity.

b. TNF is expressed on L10A2.J and other B cell lines

Membrane TNF is expressed on several cell types including activated macrophages, T lymphocytes, B lymphocytes, and certain tumor cells (known TNF-expressing cells are shown on Table 1.4, p. 9, with references). Because B cells express TNF and because NC activity is mediated by TNF, we also expect L10A2.J to express TNF. L10A2.J, RAW 8.1, WEHI 231, 2PK3 were lysed. The lysate constituents were separated on a polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-TNF antibody (Fig. 7.2). Trimeric TNF in both the membrane form (80 kDa) and the secreted form (50 kDa) were found in the B cell lines, L10A2.J (lane 4), RAW 8.1 (lane 2), WEHI 231 (lane 3), and 2PK3 (lane 5) as well as normal BALB/c mouse spleen cells (lane 7) and blood cells (lane 8). 10ME cells do not express either form of TNF (lane 6). Therefore, L10A2.J and other B cell NC effectors express both the membrane [mTNF] and secreted [sTNF] forms of TNF. Luetig et al (1989) have found surface-associated sTNF on macrophages, although the putative receptor has not been characterized. We suspect that this is also the case of sTNF on L10A2.J because supernatants of L10A2.J have no significant lytic activity (Fig. 7.3A). Alternatively, sTNF may be secreted in a limited localized space after cell-to-cell contact. Regardless of the mechanism, we find both mTNF and sTNF in B cell lines.

Poly-A⁺ RNA was isolated from L10A2.J, 10ME, and spleen cells using oligo-dT-cellulose (Fig. 7.4A). TNF mRNA could not be detected by Northern blot hybridization (Fig. 7.4B). Reverse transcriptase polymerase chain reaction [RT-PCR], a more sensitive method, was then used to test for TNF mRNA. Briefly, poly-A⁺ RNA was reverse

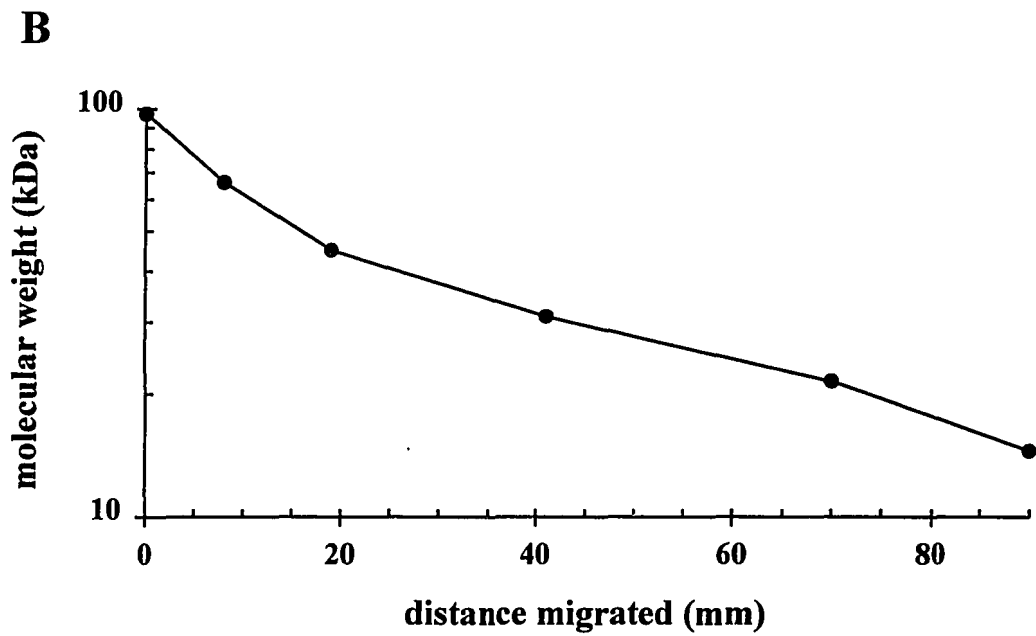
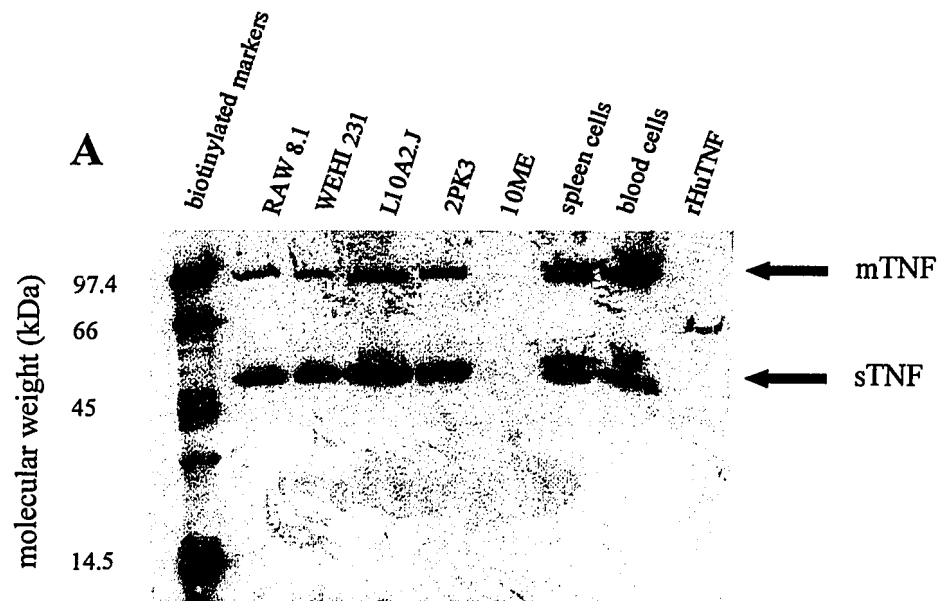


Figure 7.2. Western blot of L10A2.J using anti-TNF antibody

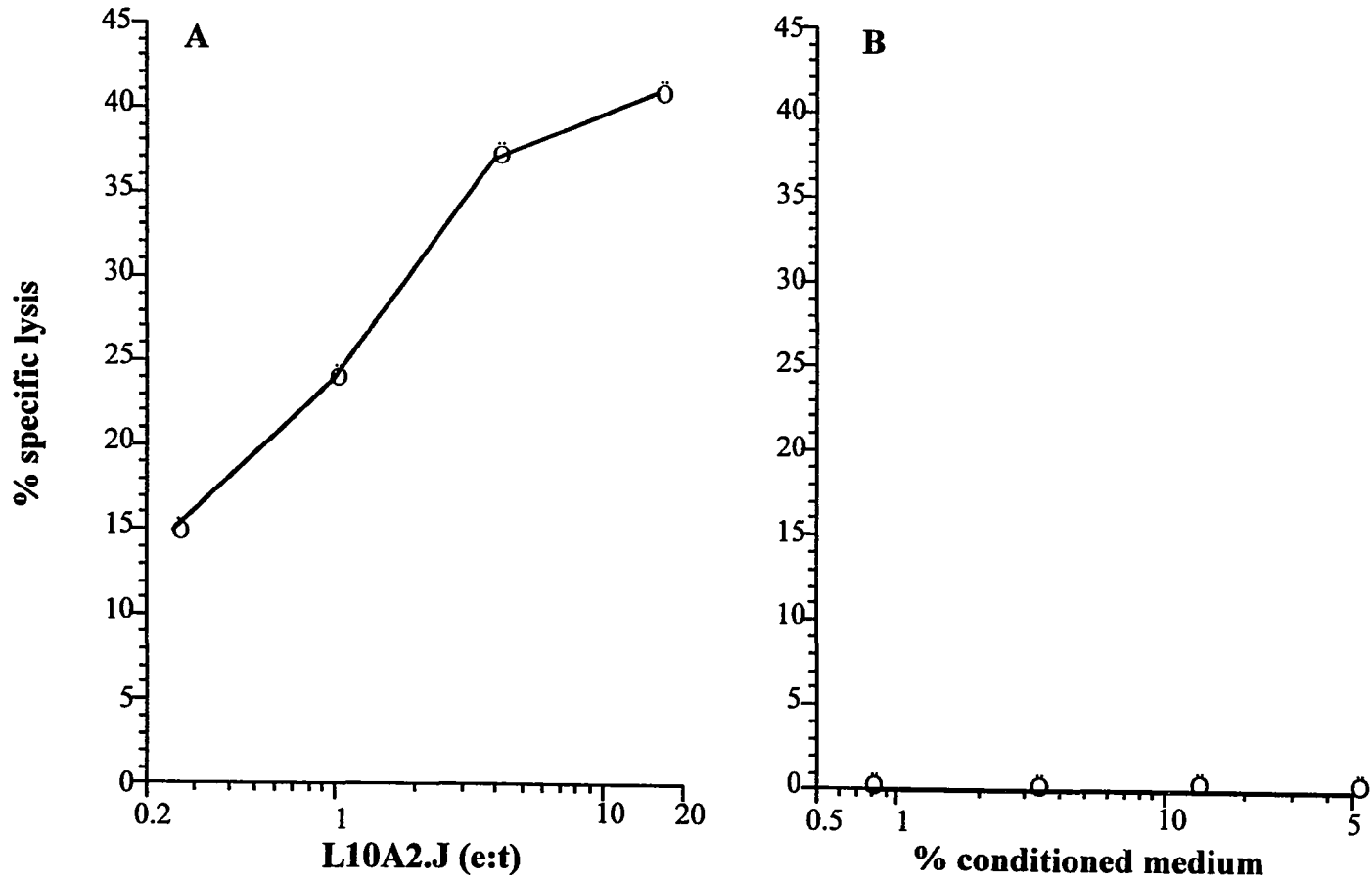
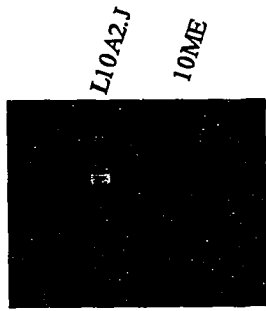
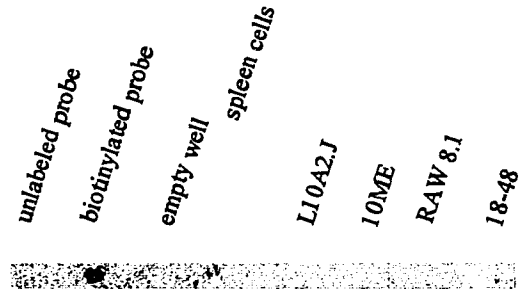


Figure 7.3. L10A2.J kills 10ME targets by a cell-associated mechanism.

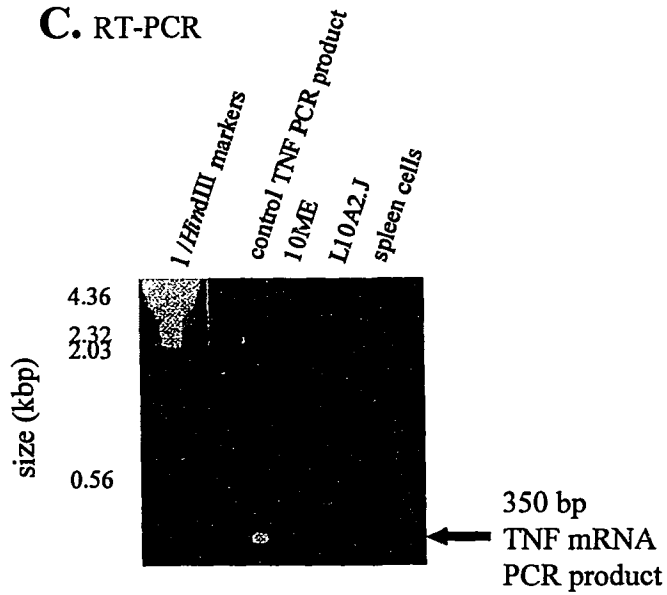
A. poly(A)+ RNA isolation



B. Northern dot blot



C. RT-PCR



D.

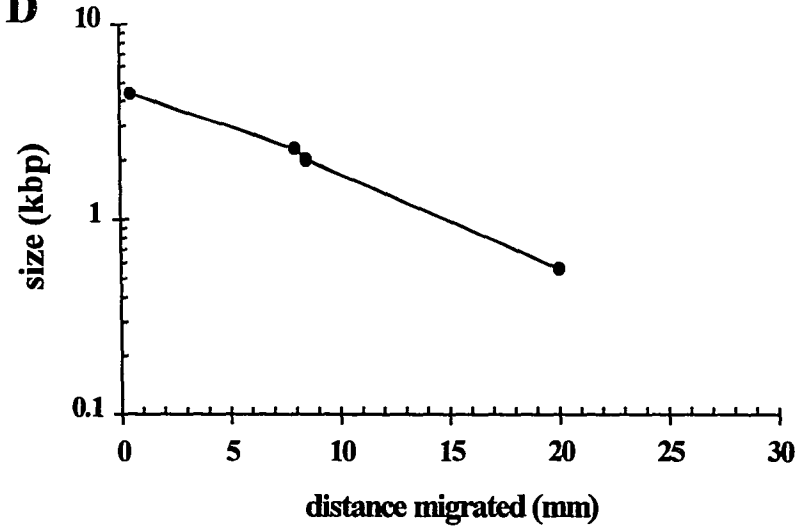


Figure 7.4. Analysis of TNF mRNA using northern blot hybridization and RT-PCR

transcribed into cDNA. This cDNA was then amplified by PCR using the following primers which amplify a 354 bp fragment (Fig 7.4C):

5' primer: 5'-TTC TGT CTA CTG AAC TTC GGG GTG ATC GGT CC-3'

3' primer: 5'-GTA TGA GAT AGC AAA TCG GCT GAC GGT GTG GG-3'

PCR product of 350 bp was found in L10A2.J (lane 5), and spleen cell mRNA (lane 6) as well as the TNF cDNA control (lane 3). No PCR product was detected in 10ME mRNA (lane 4). The expression of TNF mRNA by L10A2.J does not eliminate the possibility that LT may be involved in L10A2.J NC activity, but confirms the hypothesis that TNF is a major effector molecule in NC-mediated killing.

c. TNF plays a role in binding

TNF has a strong affinity for the TNFR. Thus, one needs to consider whether the binding of surface TNF on effectors and TNFR on targets plays a role in effector-target binding. If TNF-TNFR interactions contribute to effector-target binding then it is expected that both TNF and anti-TNF antibody would act to inhibit the effector-target interaction. Addition of TNF or antibody against TNF reduces the binding of L10A2.J to B/CN or 10ME targets (Fig. 7.5). The reduction of binding, however, is incomplete. This could indicate that TNF is one among many molecules that aid the effector in binding the target. Additionally, the TNF-TNFR interaction could be necessary to induce the surface expression of adhesion molecules. Binding induction has been demonstrated in the "leukocyte rolling" model of leukocyte attachment and migration in which cytokines first induce endothelial adhesion by selectin and the selectin-binding lectin ligand (Mackay and Imhof, 1993). Following initiation of adhesion, strong adhesion is mediated by conformational changes in adhesion molecules such as LFA-1 and ICAM-1, and $\alpha 4$ integrin and VCAM. Similar mechanisms may be operative in the NC system, too.

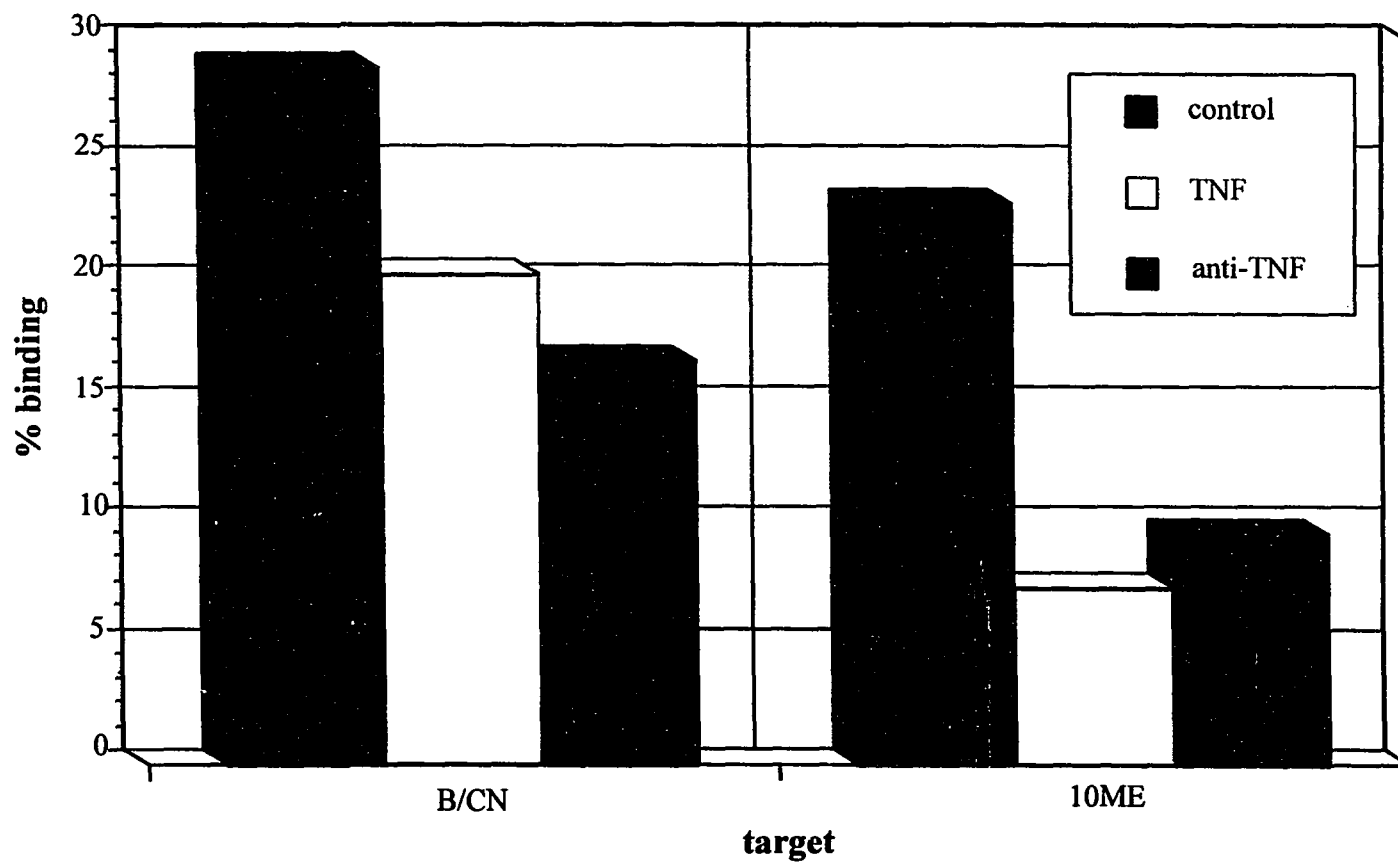


Figure 7.5. Binding of L10A2.J to 10ME targets is reduced by rTNF or anti-TNF antibody.

d. L10A2.J is not autostimulated by TNF

Macchia et al (1993) reported that B cells from HIV-infected individuals can be stimulated by TNF to grow polyclonally. Activation of normal B cells increases the expression of p75 TNFR and enhances DNA synthesis (Banchereau and Rousset, 1992). It is therefore possible that L10A2.J is stimulated by TNF.

We investigated whether TNF has a role in autostimulating L10A2.J or other B cell lines in addition to its role in binding and killing of targets by NC effectors. Exposure of L10A2.J to rHuTNF or to mitomycin-treated L10A2.J (i. e., cell-associated TNF) does not significantly increase plating efficiency in comparison to an untreated control (Fig. 7.6). Thus, it is unlikely that L10A2.J is mitogenically stimulated by TNF.

e. Conclusions

We found that 1) L10A2.J expresses TNF mRNA and both sTNF and mTNF; 2) binding and killing of 10ME targets by L10A2.J NC activity can be substantially, but not completely, reduced by anti-TNF antibody; and 3) L10A2.J NC activity is cell-associated. We interpret these data to indicate that NC effectors use cell-associated mTNF and sTNF to bind to and kill targets. A role in binding of leukocytes to adherent cells is not unique to the TNF-TNFR system; recently another cytokine-receptor system, stem cell factor [SCF] and SCF receptor, has been shown to have a role in the binding of stem cells to stromal cells (R&D Systems, 1994). It is also likely that other factors are involved in binding and killing targets by NC effectors. Fixed NC effectors express TNF, but do not kill target cells (Vanderslice and Collins, 1991). This property indicates that it is likely that other factors are also involved in NC-mediated killing of targets, and that some factors may need to be expressed upon effector cell-to-target cell contact.

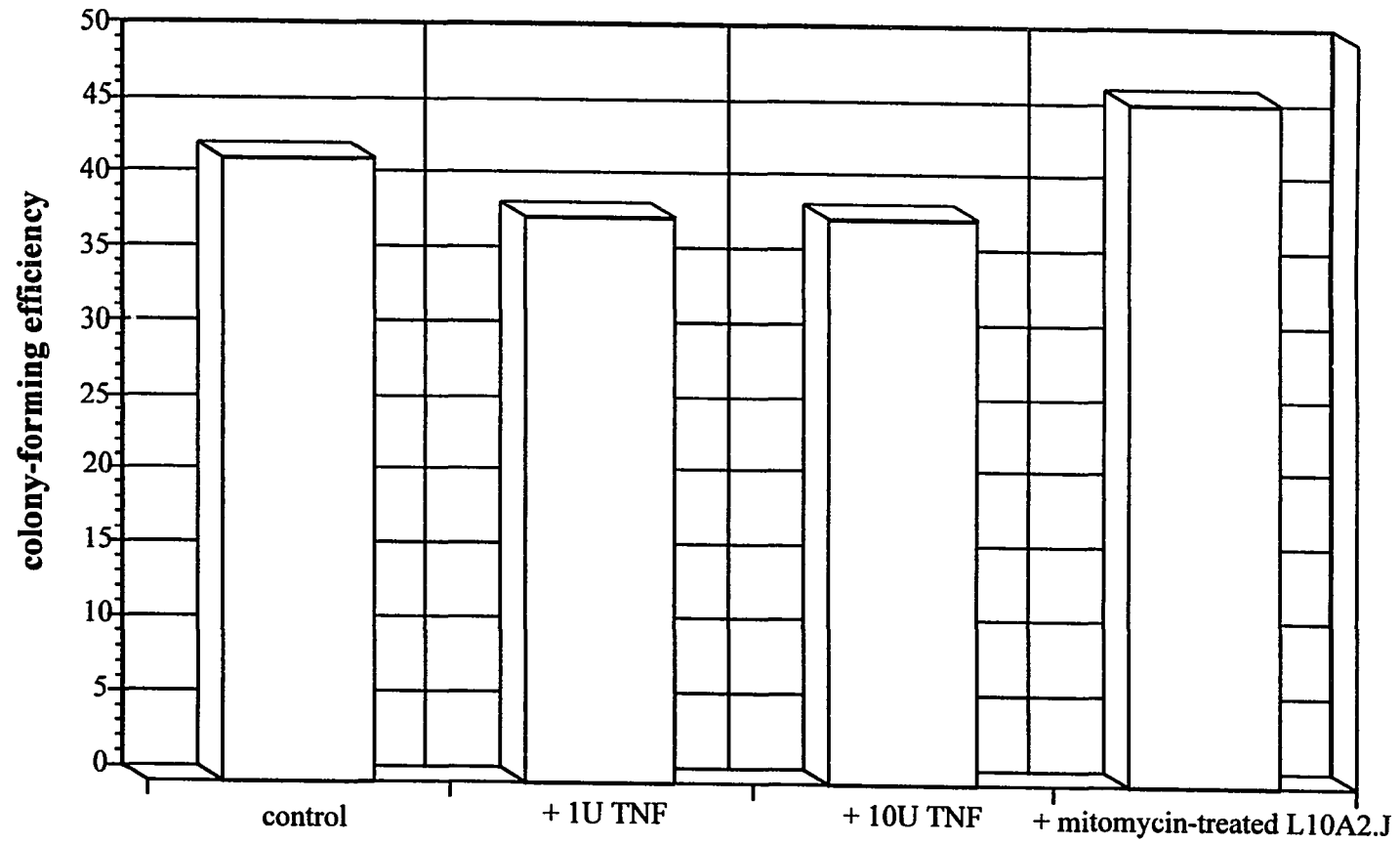


Figure 7.6. L10A2.J is not autostimulated by TNF.

Chapter 8. Other determinants are involved in the binding of NC effectors to target cells

TNF or anti-TNF antibody reduces the binding of L10A2.J NC effectors to 10ME target cells (Fig. 7.5, p. 94). Anti-TNF antibody also reduces the killing of 10ME by L10A2.J (Fig. 7.1, p.88). However, the antibody-mediated reduction in binding and killing of targets by NC effectors is not complete whereas anti-TNF antibody totally inhibits killing by rHuTNF. We interpret these data to indicate that, apart from TNF-TNFR interactions, there are additional determinants involved in binding and/or killing of targets by NC effectors.

Here we investigate whether proteins other than TNF are involved in the binding of L10A2.J to 10ME. Two approaches were used. First, we used two-dimensional polyacrylamide gel electrophoresis [2D-PAGE] to analyze the protein composition of L10A2.J and L10A2.J.sT. This demonstrated that certain proteins expressed in the NC effector, L10A2.J, are not expressed in the nonbinding L10A2.J variant, L10A2.J.sT. Finally, we examined the effect of LFA-1, a molecule commonly involved in cell-to-cell binding reactions involving leukocytes.

a. L10A2.J and L10A2.J.sT have different protein expression profiles

L10A2.J.sT is a variant of L10A2.J selected for reduced binding to the NC target, 10ME (Chapter 5). The reduced binding by L10A2.J.sT correlates with reduced killing of 10ME. We hypothesized that L10A2.J.sT has reduced expression of surface determinants that are important for binding of L10A2.J to 10ME. Total proteins from L10A2.J and L10A2.J.sT were analyzed by 2D-PAGE. Proteins were first separated on the basis of isoelectric point by isoelectric focusing [IEF], followed by separation by molecular weight in an SDS-PAGE. The density of several protein spots is diminished

for L10A2.J.sT in comparison to L10A2.J (Fig. 8.1). Thus it is possible that L10A2.J NC effectors use more than one protein to facilitate binding to 10ME targets. However, because these proteins are from whole cell lysates, these proteins are not necessarily surface proteins.

b. NK adhesion molecules as potential NC adhesion molecules

NK effectors do not kill NC targets such as 10ME and L929 cells; conversely, NC effectors do not kill NK targets such as YAC-1, K562, and Daudi cells (Lattime et al, 1981; Collins et al, 1981). However, killing of NC targets by spleen cells can be competed by NK targets (Lin et al, 1983; Collins et al, 1986). This competition implies that NC effectors and targets share recognition determinants, but not lytic mechanisms, with NK effectors and targets.

Numerous surface proteins and glycoproteins have been described on B lymphocytes (Table 8.1). Several cluster of differentiation (CD) molecules as well as cell adhesion molecules [CAM] have adhesion properties (Table I) (Dustin and Springer, 1991; Banchereau and Rousset, 1992). Although numerous other surface molecules could be important for adhesion of B cells to other cells, we focus on those molecules whose primary role appears to be cell-to-cell adhesion. Analysis of the adhesion molecules of B lymphocytes has revealed several functions. First, adhesion molecules facilitate the migration of B lymphocytes (Mackay and Imhof, 1993). B lymphocytes attach to cells lining the vessels or organs (e.g., lymph nodes) and begin rolling via L-selectin. LFA-1 and integrins facilitate strong adhesion to target tissue. Adhesion molecules are also important in the T cell-B cell interaction in both antigen presentation by B cells, and in T helper stimulation of B cells (Dustin and Springer, 1991; Parker, 1993). LFA-1 found on both the T cells and the B cells forms a strong adhesion with ICAM-1 found on T cells and B cells. Also, LFA-3 on B cells binds to CD2 on T cells.

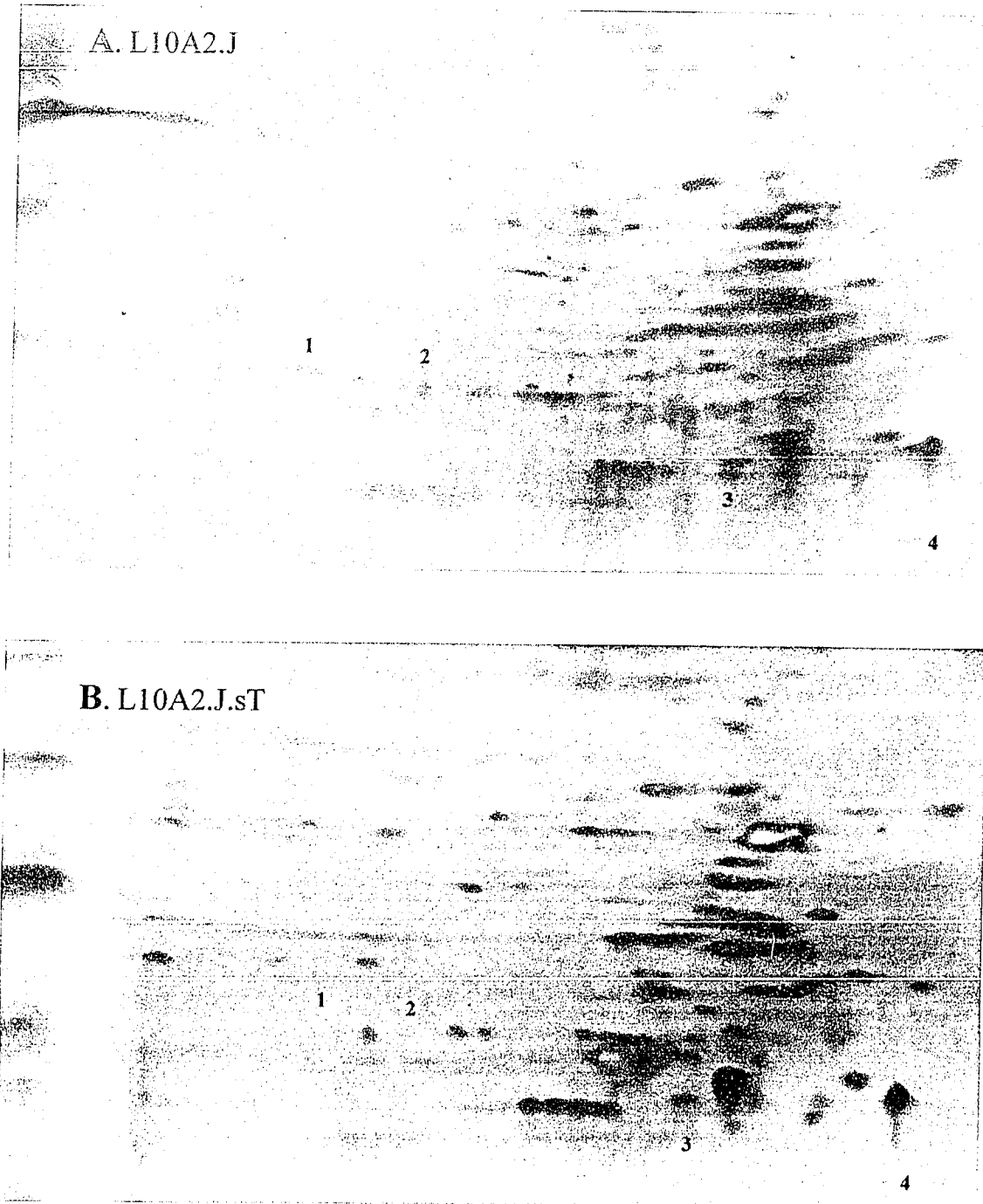


Figure 8.1. 2D-SDS-PAGE of L10A2.J and L10A2.J.sT. Numbers denote proteins significantly stronger in L10A2.J than L10A2.J.sT.

Table 8.1. B cell adhesion molecules

adhesi mol.	structure	B cell type	ligand	ligand found on	function
ICAM-1†* (CD54)	Ig domains, Ig superfamily	peripheral B	LFA-1	most leukocytes	strong adhesion to leukocytes and blood endothelium
ICAM-2†*	Ig domains, Ig superfamily	circulating B	LFA-1	most leukocytes	strong adhesion to leukocytes and blood endothelium
ICAM-3†*	Ig domains, Ig superfamily	all B	LFA-1	most leukocytes	strong adhesion to leukocytes and blood endothelium
LFA-3†* (CD58)	Ig domains, Ig superfamily homology to CD2	all B	CD2	T cells	T cell adhesion
LFA-1†* (CD11 + CD18)	β2 integrin	most B	ICAM-1,-2,-3	leukocytes, endothelium	strong adhesion to leukocytes and blood endothelium
VLA-1†*	β1 integrin	activated B	laminin, collagen	extracell. matrix	strong adhesion to ECM
VLA-2†*	β1 integrin	activated B	laminin, collagen	extracell. matrix	strong adhesion to ECM
VLA-4†*	β1 integrin	some resting B	fibronectin VCAM-1	extracell. matrix follic. dend. cells	strong adhesion to ECM and to dend. cells
VLA-5†*	β1 integrin	some resting B	fibronectin	extracell. matrix	strong adhesion to ECM
L-selectin†* (LECAM, LAM-1, MEL-14, Leu-8)	selectin	most B	PLN addressins	endothelial cells	lymph node homing
CD22* (α and β)	heterodimer Ig-like domains, homol. to V/N-CAM	mature B	CD45 RO	CD4 T cells	? stimulatory signal to B cells and activation of T cells
CD44* (PGP-1)	heavy glyco., 90 kDa, homol. to cartilage link protein and proteoglycan	all types	hyal. acid vasc. addressins	extracell. matrix mucosal cells	lymphoc. trafficking and endoth. cell recog.; reg. tumor develop.; reg. CD2/LFA-3 adhes.
CD48* (Blast-1)	43 kDa glycoprot., Ig superfamily	activated B	CD48	activated B, T cells, monocyte	adhesion

†: data from Dustin and Springer (1991); *: data from Banchemau and Rousset (1992)

There is also evidence that B cell-B cell adhesion is facilitated by LFA-1 binding ICAM-1. The reason for this is unclear although it is possible that some homotypic regulation is occurring (Dustin and Springer, 1991). Finally, at least one adhesion molecule, CD44, appears to have multiple functions including a tumor regulatory role.

The expression of many of these adhesion molecules and ligands is linked to the stage of development or state of activation of the B cell. Therefore, one can speculate that most cytokines that affect differentiation or activation of B lymphocytes will also affect their adhesion molecules. CD40, a receptor of the TNFR/NGFR family causes an increase in B cell proliferation including an increase in adhesion mediated via LFA-1 and LCAM-1 (Banchereau and Rousset, 1992; Clark and Ledbetter, 1994). Likewise expression of adhesion molecules or ligands to B cell adhesion molecules that are found on target cells are affected by cytokines. For example, cytokines secreted by monocytes can increase expression of ICAM-1 or ICAM-2 (Dustin and Springer, 1991). In particular, IFN-g stimulates ICAM-1 expression, and TNF in conjunction with IFN-g can further stimulate ICAM-1 expression (Horejsi, 1991; Vassalli, 1992).

The B cell adhesion molecule, LFA-1 has also been identified as NK adhesion molecules (Table 8.2). Because NC and NK targets can compete for NC effectors, we investigated the role of LFA-1 in binding of NC effectors to targets (Fig. 8.2). Antibodies against LFA-1, MHM23 and MHM24 (a generous gift from A. J. McMichael), reduce binding of L10A2.J to 10ME. We interpret the incomplete inhibition of binding to indicate that other adhesion molecules are involved in NC cell-target cell binding. Multiple adhesion interactions are known to be important for effector-target binding in NK- and CTL-mediated killing as well as B cell-T cell interactions (Storkus and Dawson, 1991; Karre et al, 1991; Clark and Ledbetter, 1994). In general, LFA-1 appears to play a central role in all of these actions.

Table 8.2. NK cell adhesion molecules

adhesion molecule	found on B cells?	ligand	NK-related function	ref
N-CAM	n.d.	N-CAM	adhesion	1-3
FAM	n.d.	unknown	potential NK receptor	4-5
fibronectin	n.d.	VLA-4, -5	adhesion, upregulated upon activation	1,3-4
laminin	n.d.	VLA-6	adhesion, upregulated upon activation	4
LFA-1	yes	ICAM-1, -2, -3	adhesion to all targets	1,3,6
CD2	no	LFA-3	adhesion in some targets, activation in some targets	1,3
NK-R1	n.d.	unknown	potential NK receptor	4
NKR-P1	n.d.	unknown	potential NK receptor	4,7-8
NKG2	n.d.	unknown	potential NK receptor	7
Ly-49	n.d.	MHC class I	MHC class I allo-specific NK receptor	7,9

references

¹ Timonen et al, 1990

² Nitta et al, 1989

³ Storkus and Dawson, 1991

⁴ Karre et al, 1991

⁵ Evans et al, 1993

⁶ Schmidt et al, 1985

⁷ Hofer et al, 1992

⁸ Ryan et al, 1991

⁹ Karlhofer et al, 1992

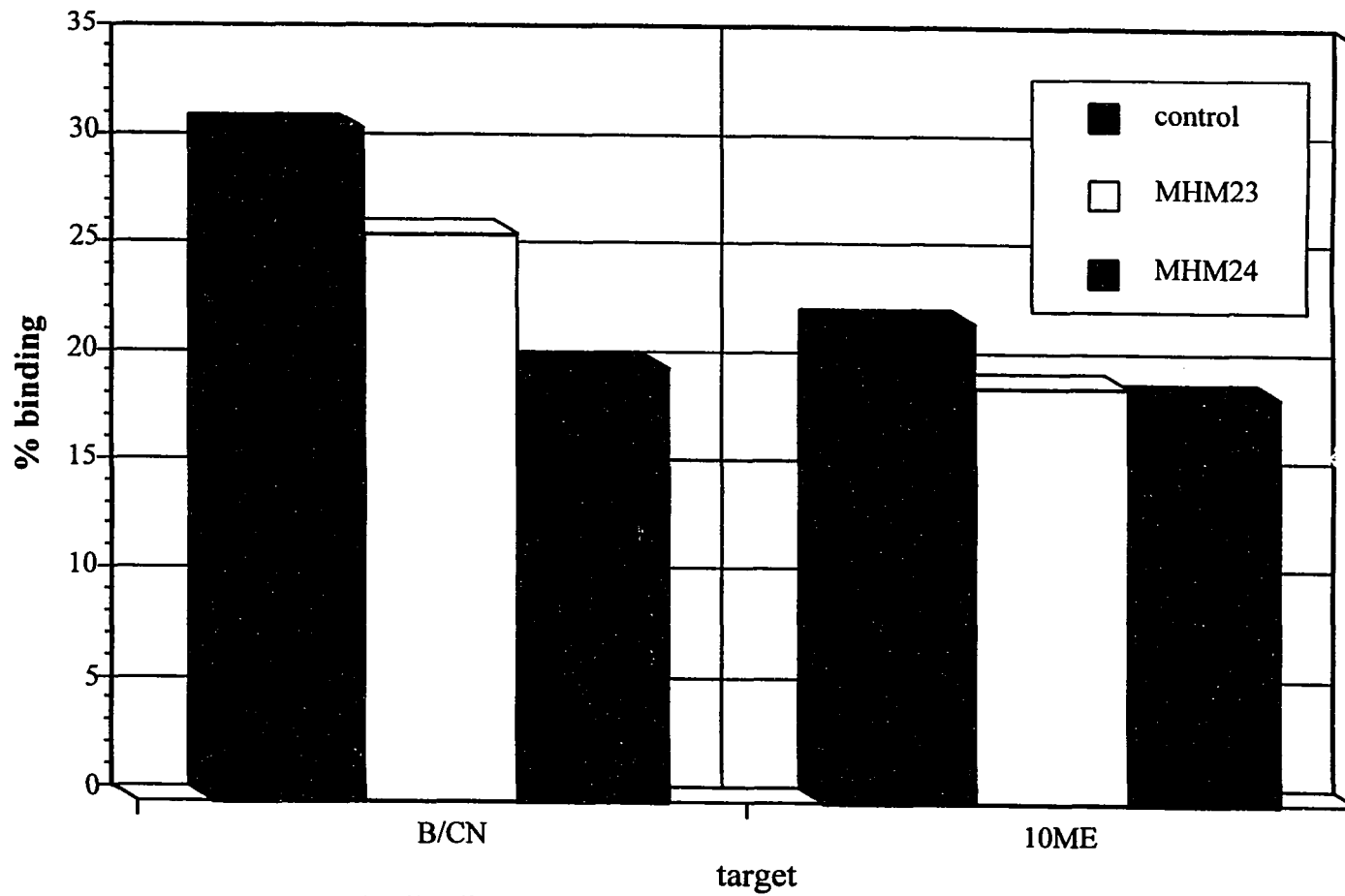
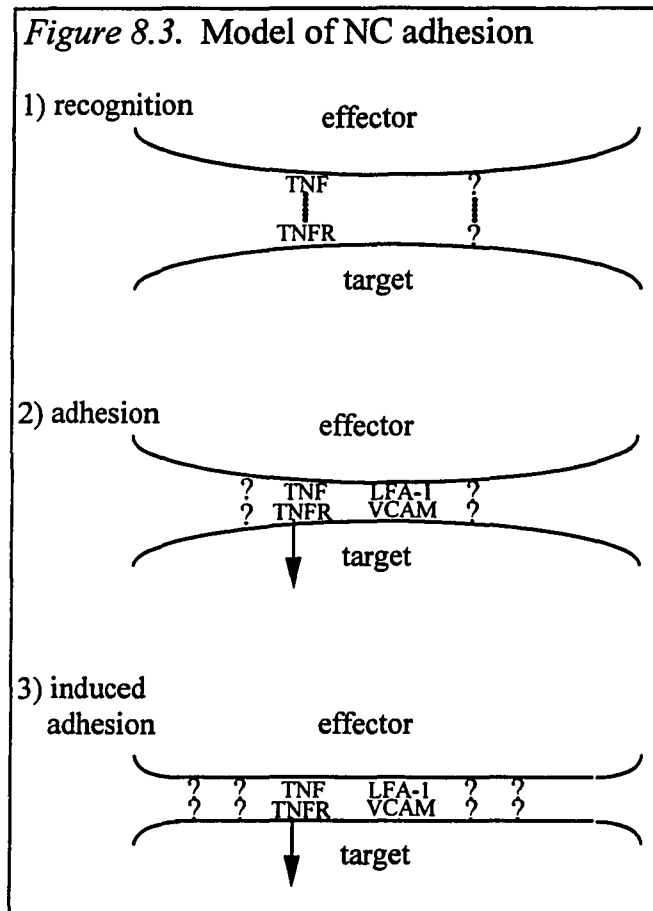


Figure 8.2. Antibodies against LFA-1 reduces binding of L10A2.J to 10ME.

c. Conclusions

Binding of NC effectors to targets involves both TNF and perhaps LFA-1, and probably involves other adhesion molecules. A crude model of NC effector-target binding is similar to other leukocyte interactions with other cells (Fig. 8.3). First, an immediate recognition and weak binding occurs; this may involve the TNF-TNFR interaction. Immediately, a number of strong adhesion interactions are formed to strengthen cell-to-cell contact; the LFA-1-VCAM interactions are both numerous and strong enough to form the firm contact. This is possibly further strengthened by induced surface expression of more adhesion molecules such as the fibronectin-VLA, laminin-VLA-6, and CD45-CD45L interactions of NK effectors and targets. While the adhesion interactions are occurring, the TNF-TNFR interaction initiates the lytic and counterlytic signals which may result in the apoptotic death of the target cell.

Because NC- and NK-mediated killing of targets both involve LFA-1 (and probably other adhesion determinants), we observe the competition of NC and NK targets for NC effectors.



Chapter 9. Conclusions

Cancer is the second leading cause of death in the United States and other developed countries (W.H.O., 1990; Boring et al, 1993). Progress towards a general cure for cancer has been slow. Mortality due to specific types of cancers have generally decreased due to the progress of surgical therapy, radiation therapy, and chemotherapy. In the majority of cases, however, the most effective of these treatments are either invasive or have a high level of toxicity to the patient. Much recent attention has focused on biological therapies such as hormone based treatment or immunotherapy, because these modalities offer a lower risk of side effects.

The human immune system has evolved as an effective system for the clearance of infectious diseases. We now suspect that it has also evolved as an effective system for the surveillance of tumors (Burnett, 1970). Among the mediators of immune surveillance of tumors is the natural cytotoxic [NC] cell. In the model developed by Patek et al (1978), NC cells are able to kill cells that have become transformed. In order for the transformed cell to develop into a tumor, it must gain the ability to escape surveillance by NC cells.

The research presented in this dissertation has uncovered novel and significant properties of NC cells and their targets. In particular, three significant properties were unveiled by our research. First, most transformed B lymphocyte cell lines are NC effectors. Also, binding of NC effectors to targets is important to killing. Finally, the pleiotropic cytokine, tumor necrosis factor [TNF], is important, but not exclusively involve in the binding of NC effectors to targets.

a. L10A2.J and other transformed B cell lines are cloned NC effectors

L10A2.J is a B cell line that is phenotypically similar to splenic NC effectors (Chapters 3 and 4). L10A2.J is able to kill NC-sensitive targets, but not NC-resistant

targets, in an 18-hour cytotoxicity assay. In addition, L10A2.J-mediated killing is not similar to NK-mediated or CTL-mediated killing and does not require immunoglobulin or stimulation with LPS. Killing of targets by L10A2.J requires effector-target contact and secreted factors play little or no role in the cytotoxicity. Furthermore, TNF is involved in the killing of target cells by L10A2.J in a manner consistent with the apoptotic death induced by soluble TNF alone. Thus, L10A2.J probably uses a cell-associated TNF to kill targets. We confirmed the presence of TNF mRNA as well as two membrane and secreted forms of TNF in L10A2.J (Chapter 7). In addition, 10ME cells selected for resistance to L10A2.J behave like 10ME cells selected for resistance to spleen cells and 10ME cells selected for tumorigenic growth. Each of these cell lines is resistant to spleen cell- and L10A2.J-mediated NC activity and is able to grow as a tumor in normal, immunocompetent mice. We can use L10A2.J in place of heterogeneous spleen cell populations to study specific cellular and molecular properties of NC effectors.

Most other transformed B cell lines also have NC activity (Chapter 4). Although these cell lines have not been as extensively tested as L10A2.J, there are compelling reasons to believe that they, too, can serve as cloned NC effectors. These cells kill 10ME in an 18-hour cytotoxicity assay by a cell-associated mechanism. Killing by the B cell lines, RAW 8.1 and WEHI 231, also require TNF which is expressed by the cells. B cells isolated from spleen also have NC activity. Furthermore, Lopez-Cepero et al (1994) have recently demonstrated that normal B cell populations can kill NC-sensitive targets and produce TNF. It is reasonable, therefore, to assert that some B cells are NC effectors.

b. Binding is important to killing of targets by NC effectors and important for the Immune Surveillance of tumors

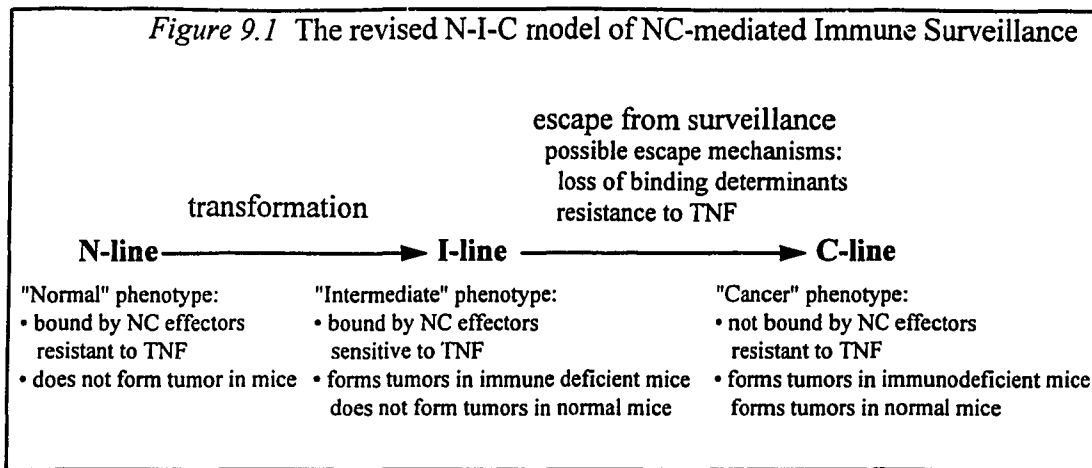
Binding of NC effectors to targets is important for killing of the targets (Chapter 5). NC effectors selected for reduced binding to targets also have reduced cytotoxicity to

target cells in vitro. Therefore, NC effectors need binding and cytotoxic mechanisms to carry out their function.

Binding of NC effectors to targets is also important for the Immune Surveillance of tumors (Chapter 6). NC effectors are able to kill transformed (I-line) targets, but not tumorigenic (C-line) targets. In this investigation, we found that NC effectors bind both N-line and I-line target cells efficiently. However, C-line cells, derived from I-line cells via selection for resistance to NC activity or selection for tumorigenicity in normal mice, are not efficiently bound by NC effectors.

Using the information that we have obtained in this study, we have modified the N-I-C model of NC-mediated Immune surveillance (Fig. 9.1). We suggest that most cells can be bound by NC effectors. It is likely that cells bound by NC effectors are exposed to a TNF signal, but in N-lines the protective signal is strong enough to counteract any apoptotic signal. Although this process would need a lot of NC effectors, we believe that this is consistent with the fact that NC effectors may be found in nearly all leukocyte populations (Lattime et al, 1981). If this is true, it is likely that NC activity is very ancient. It is tempting to speculate that TNF-susceptibility is linked to the deregulation of growth due to the transformation event. Thus, transformation leads to cells that are susceptible to TNF-mediated lysis by NC effectors (i. e., the I-cells). As with N-lines, we assume that I-lines are surveyed by NC effectors, except the I-lines are susceptible to TNF and thus eliminated. There is evidence that the susceptibility to TNF is due to a loss of protective mechanisms (C. Sasaki and P. Patek, personal communication). C-line cells gain, through somatic mutation, the ability to escape immune surveillance. We interpret our data to indicate that C-lines escape surveillance by reduced expression of binding determinants and thus become resistant to TNF and NC-mediated lysis.

Figure 9.1 The revised N-I-C model of NC-mediated Immune Surveillance



c. *TNF and LFA-1 are important for binding of NC effectors to targets*

The final point addressed by this dissertation is whether TNF has a role in binding of NC effectors to targets and whether it is the sole mediator of binding. We found that NC effectors require TNF for binding as well as for cytotoxicity (Chapter 7). It is not unprecedented that cell-associated hormone contributes to effector-target binding; Simmons (R & D Systems, 1994) has determined that another cytokine, stem cell factor, has a dual role in stem cell mitogenesis and in the binding of stem cells to stromal cells. Because binding of NC effectors to targets is not completely abrogated by anti-TNF antibodies or by rHuTNF, TNF alone can not solely account for all binding of NC effectors to targets.

Indeed, several proteins have reduced expression in NC effectors selected for reduced binding (Chapter 8). Furthermore, there are several adhesion molecules known to be expressed on B lymphocytes and several have been described for NK cells. Because NC-mediated cytotoxicity can be competed by NK targets, but NC effectors don't kill NK targets, Lin et al (1986) have proposed that NC and NK effectors share recognition determinants, but do not share lytic mechanisms. We determined that LFA-1, an adhesion molecule found on NK cells and found on B lymphocytes, is also important in binding of NC effectors to targets.

d. NC activity and the cure for cancer

Our knowledge of NC activity is still emerging. Although we are far from bringing about any novel NC-associated treatments for cancer, we have done some essential basic research in this area. The study presented in this dissertation has provided us with cloned NC effectors to use as tools for further NC research and has given us a better understanding of how NC effectors interact with targets.

One of the major problems of cancer immunotherapy is the nonimmunogenicity of tumors. Several approaches with tumor-infiltrating lymphocytes [TIL] have shown preliminary success probably due to the TIL specificity for a tumor (Rosenberg et al, 1986). Other approaches involve the transfer of cytokine or histocompatibility genes to boost the cytotoxic T lymphocyte response against tumors (Colombo and Forni, 1994). Understanding of the binding and lytic mechanisms of NC activity can lead to therapeutic methods similar to those used in TIL and cytokine gene transfer therapies. One of the advantages of such immunotherapies is that they would allow the patient's own immune system to fight the cancer and would not result in the toxicity of chemotherapy nor the invasiveness of surgical therapy. In any case, because no substantially successful cures have yet been developed for cancer, all potential lines of research should be investigated.

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