"THE ANTIBODY RESPONSE TO TUMOR SPECIFIC ANTIGENS"

by

JAMES DALTON

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

© 1975

Permission has been granted to the LIBRARY OF THE UNIVERSITY OF MANITOBA to lend or sell copies of this dissertation, to the NATIONAL LIBRARY OF CANADA to microfilm this dissertation and to lend or sell copies of the film, and UNIVERSITY MICROFILMS to publish an abstract of this dissertation.

The author reserves other publication rights, and neither the dissertation nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

ACKNOWLEDGEMENTS

A special thanks to Professor A. Sehon, who made my studies in this department possible, for his enthusiasm, for his guidance, and for his many helpful discussions.

Thanks also to Dr. S. Fujimoto for his untiring patience and encouragement in directing this work.

I am indebted to the University of Manitoba for the award of a Manitoba Fellowship during my work in this department, and to Professor Sehon for the provision of research and travel funds.

46638

ABSTRACT

Antibodies specific for tumor antigens have been successfully induced in syngeneic (A/J), and in H-2 compatible allogeneic mice (B10·A). Cultured cells of the spontaneous A/J lymphoma L1117 were used to immunize A/J and B10·A mice. Each animal received biweekly injections of 10^6 mitomycin C-treated L1117 cells i.p. and was partially bled from the tail vein prior to each injection. In both strains the antibody response was shown to be specifically cytotoxic for L1117 cells, and no reduction in titre could be achieved by incubation with normal lymph node cells, normal thymus cells, 1509a fibrosarcoma cells or fetal cells of A/J mice. Antibodies from serum of both strains were shown to bind specifically to L1117 cells by using fluorescein-labeled rabbit anti-mouse $F(ab')_2$ for indirect cell membrane staining.

Antibodies were present at a low level in the first test bleeding at 2 weeks and continued to rise in titre until week 10 in the B10·A strain and until week 14 in the A/J strain. The response of A/J mice was markedly slower, the maximum titre reached in both strains being approximately the same. The classes of antibodies produced were somewhat unusual, both strains producing predominantly IgM antibodies even after 20 weeks immunization while IgG antibodies accounted for only a small part of the cytotoxic activity.

It is suggested that the tumor antigen (TA) may act as a "thymus-independent" antigen, thus the weak 19S response is the maximum possible in the absence of T cell cooperation. Comparison with results obtained elsewhere for the pneumococcal polysaccaride

type III antigen (SIII) further suggest that TA might activate "suppressor" T cells which actively prevent a normal antibody response.

The ability of normal A/J and B10·A mice to reject a challenge with graded doses of viable L1117 cells was taken as a measure of their capacity to mount a cell-mediated response to TA. No evidence of cell-mediated immunity was observed in strain A, all tumor cell doses tested being rapidly fatal to the mice. In contrast B10·A mice rejected the same challenge doses in all cases. The parallel between the ability of immunized B10·A mice to produce 7S antibody and the ability of normal B10·A mice to reject viable tumor cells may indicate that in this strain TA of L1117 cells activates helper T cells to a greater extent than in strain A mice.

TABLE OF CONTENTS

CHAPTER		PAGE
I	INTRODUCTION	1
II	REVIEW OF PERTINENT LITERATURE	6
	1. Tumor cell Antigens	7
	2. Host Responses To Tumor Antigens	
	A. Cellular Responses	10
	B. Antibody-mediated Responses	13
	3. Role Of The Immune Response In Tumor Growth	
	A. General consideration of mechanisms	23
	B. Role of antibody	26
	(i) Promotion of tumor growth	26
	(ii) Inhibition of tumor growth	29
	(iii) Antigenic Modulation	30
	4. Summary	32
III	NATURE OF THE PROBLEM	34
IV	MATERIALS AND METHODS	38
V	RESULTS	47
	1. Specificity Of The Antibody Response	48
	A. Specificity for tumor antigens	49
	B. Recognition of fetal antigens	54
	2. Kinetics Of The Antibody Response	57
	3. Antibody Characterization	59
	4. Tumor Growth *in vivo*	67
VI	DISCUSSION	70
	1. Specificity Of The Antibody Response	71

CHAPTER PAGE

A. Specificity for tumor antigens 71

B. Cross-reactivity with fetal antigens 75

2. Nature Of The Antibody Response

A. Kinetics of the antibody response 77

B. Significance of the 19S response 81

3. Conclusions 86

REFERENCES 87

APPENDIX I - THE TWO-STEP CYTOTOXICITY TEST 98

APPENDIX II - A STATISTICAL EVALUATION OF THE CYTOTOXICITY TEST DATA 103

LIST OF ILLUSTRATIONS

FIGURE		PAGE
1.	Specificity of cytotoxic activity	50
2.	Absorption of cytotoxicity by incubation with cells	51
3.	Specificity of immunofluorescent staining	52
4.	Absence of activity toward fetal cells in B10·A anti-L1117 antiserum	55
5.	Kinetics of the antibody response	58
6.	Characterization of cytotoxic antibody in B10·A antiserum	60
7.	Characterization of cytotoxic antibody in A/J antiserum	61
8.	Characterization of cytotoxic antibodies in serum fractions by mercaptoethanol resistance.	63
9.	Comparison of cytotoxicity tests	101

TABLE		
1.	Determination of antibody classes with ^{125}I-labeled anti-immunoglobulins	65
2.	Cell-mediated response to tumor antigens	68
3.	Lysis of L1117 cells by anti - L1117 antiserum	105

CHAPTER I

INTRODUCTION

Considerable evidence exists indicating that malignant cells possess specific antigenic determinants which appear as new antigenic moieties on cells transformed by viruses (6,7) or chemical carcinogens (4,5). Moreover it has been shown repeatedly that the tumor-bearing host is capable of recognizing these new antigens and producing a demonstrable response. As early as 1953 Foley (1) used tumor ligation and release to immunize the autochthonous host against subsequent tumor challenge, work that was substantiated by the classic experiments of Prehn and Main (2) and by the work of G. Klein and his associates (3). These experiments used rejection of tumor transplants to show immunity, consequently the antigens so identified became known as tumor-specific transplantation antigens (TSTA). Since that time a variety of *in vivo* and *in vitro* assays have been used, and have given rise to the more general designations tumor associated antigen (TAA) and/or tumor specific antigen (TSA). This report deals with the antibody response to an antigen present on murine lymphoma cells which cannot be detected on normal cells of the same strain, so the term tumor antigen (TA) has been chosen for use throughout as a general term including TSTA, TAA and TSA.

Thymus derived lymphocytes (T cells) have been regarded as primarily responsible for tumor rejection, and it has been shown that immunity to a chemically-induced tumor could be transfered with cells (4). Antibodies have traditionally been viewed as antagonistic to cell-mediated defenses because of their ability to enhance tumor growth or graft survival (35).

Reports of enhancement of tumor graft survival by antibody led

the Hellströms (29-32) to search for a similar effect protecting growing tumors in vivo. Initial reports seemed to indicate the existance of a "blocking antibody" in the circulation of tumor-bearing subjects which could specifically block their immune lymphocytes from destroying tumor cells. Later studies into the nature of the blocking factor suggest antigen (TA) or antigen-antibody complexes as being responsible.

Antibody directed to tumor specific antigens may also be cytotoxic under the appropriate conditions, and has been used to passively immunize mice against a syngeneic Gross virus induced tumor (38). In other systems a cytotoxic antibody response has been observed during progressive tumor growth, and no satisfactory explanation has been proposed for its lack of effectiveness (36).

Recent reports of antibody dependent cell-mediated cytotoxicity (ADCC) suggest another way in which antibody may be involved in the host response to tumors. It has been shown that antibodies directed to TA can potentiate tumor cell destruction by normal lymphocytes (43). Several cell types may be capable of participating in this phenomenon including macrophages and monocytes, possibly B cells, and a non-T, non-Ig-bearing cell tentatively designated a K cell (45).

Despite the potential for tumor destruction indicated by the presence of cytotoxic T cells, cytotoxic antibodies, and ADCC, neoplastic growths are often fatal to the host. Some progress is being made toward elucidating the processes regulating the immune response, notably in the area of regulatory T cells. Much work remains however, and the role of antibody in promoting or opposing tumor growth is not

yet clear.

Two possible modes of tumor destruction, cytotoxic antibodies and antibody dependent cellular cytotoxicity, have already been mentioned. Promotion of tumor growth could occur through a blocking action similar to that envisioned by the Hellströms (29-32); or by alteration of the tumor cell itself through the mechanism of antigenic modulation (76); or by antibody mediated suppression of the immune response (35). The point of action of the latter is still unknown but several reports have appeared in which specific antibody, passively or actively induced, suppressed antibody formation (35), or delayed hypersensitivity (68) in a highly specific manner. High affinity antibody produced in response to tumor antigens may be present in quantities too small to destroy tumor cells yet sufficient to suppress further antibody production (67). The combined effects of antibody mediated suppression and antigen-mediated suppression may then in turn eliminate cellular cytotoxicity (70) allowing tumor growth.

Such hypotheses must be modified or discarded as new data become available, however the fact of antibody involvement in tumor immunology is evident despite uncertainty as to its role. The present study was undertaken in order to examine the nature of the antibody response to tumor antigens and some properties of the antibodies produced.

The study reported here examines the antibody response to tumor antigen(s) of a murine lymphoma in two strains of mice, one allogeneic and the other syngeneic to the tumor. The model chosen for study employs a lymphoma of A/J mice discovered in this laboratory and designated L1117, and examines the humoral immune response in mice of A/J

and B10·A strains. Mice of strain B10·A are congenic with those of C57Bl/10J, but carry the H-2 locus of strain A. Viable tumor cells were treated to render them incapable of replication then used to immunize both strains of mice by identical protocols. The results indicate that both strains respond to the tumor cell antigen(s) in a highly specific manner, producing an antiserum which will lyse L1117 lymphoma cells in the presence of complement but having no activity against normal A/J cells nor against an unrelated tumor. Both antisera were analyzed as to kinetics of response, specificity of cytotoxic activity, immunoglobulin class(es) involved, and reactivity with antigens on fetal cells of the A/J strain.

CHAPTER II

REVIEW OF PERTINENT LITERATURE

1. TUMOR CELL ANTIGENS

The relevance of immunology to neoplasia hinges upon the existence of tumor antigens (TA), new cell surface antigens which did not exist on the tissue from which the tumor arose. The existence of such antigens was first proposed many years ago, but it did not gain general acceptance until the advent of inbred strains permitted transplantation of tumors between genetically identical animals. Foley (1) gave the first clear demonstration of tumor-specific immunity utilizing a methylcholanthrene-induced sarcoma in an established strain of inbred mice. Ligation and release of a growing tumor was followed by challenge with a graft from the same tumor. Animals rejecting this graft showed resistance to subsequent challenges with viable cells from the same sarcoma but not against unrelated tumors. Prehn and Main (2) confirmed and extended Foley's work. To eliminate any possibility that genetic differences were responsible for Foley's observations they repeated a similar series of experiments testing syngeneity by reciprocal skin grafting and demonstrating that preimmunization with tumor did not produce resistance to grafts of donor skin nor did skin grafts protect against subsequent tumor challenge. Any remaining doubts about minor antigenic differences due to heterozygosity of inbred strains were removed by the work of Klein and his coworkers (3) who immunized animals with irradiated cells from their own primary tumor.

Animal studies have indicated that spontaneous tumors, virus-induced and chemically-induced tumors all possess TA, but with one basic difference. Chemically-induced tumors each have a distinct antigen which does not cross-react even with tumors induced by the same

chemical (4,5). Virus-induced tumors also possess a distinct TA but in addition exhibit a virus-specific antigen which is cross-reactive for all tumors induced by that virus (6,7). Presumably "spontaneous" tumors would belong to one of these two groups.

Of particular interest in the present investigation are studies of viral and cell-surface neoantigens associated with virus-induced murine leukemias since preliminary work, published earlier, indicated a viral etiology for the L1117 lymphoma (8). Pasternak (9) studied virus-associated antigens in a myeloid Graffi leukemia. He found that Landschütz sarcoma I cells, which naturally harbour the virus, were capable of removing virus-neutralizing activity upon incubation with Graffi immune serum but did not decrease its ability to stain Graffi virus-infected cells in the indirect immunofluorescence test. In contrast, the original myeloid Graffi leukemia cells removed both virus-neutralizing and staining ability from Graffi immune serum. This would indicate that the original leukemia cells possessed two distinct neo-antigens, one of which was a viral antigen and the other a surface membrane antigen expressed by cells transformed by the virus.

Aoki et al. (10,11) found similar results in the case of Gross leukemia cells. Studying E ♂ G2 leukemia cells with anti-ferritin hybrid antibodies and electron microscopy they were able to show that Gross cell surface antigen, specified by Gross virus, was present on infected cells, but neither the Gross cell surface antigen nor a number of normal alloantigens were present on the viral envelope. They also noted that antiserum produced in the highly resistant C57Bl/6 strain reacts only with infected cells,

not viral envelope, and is deficient in virus neutralizing activity, probably indicating a specificity for Gross cell surface antigen. This is in contrast to the considerable neutralizing ability of antisera to Friend, Moloney, Rauscher, or Graffi leukemia virus prepared in a similar manner. The authors speculate that the lack of anti-virus antigen activity may be related to the fact that only Gross virus has been convincingly implicated in naturally occuring mouse leukemia.

Thus it appears that virus-induced murine leukemias may express two distinct types of tumor-associated antigen: a virus-specified cell surface antigen; and a virion antigen. Antisera to these leukemias could recognize either or both of these antigens and would still be considered tumor-specific in that they recognized no antigens present on normal cells of the same mouse strain.

2. HOST RESPONSES TO TUMOR ANTIGENS

A. CELLULAR RESPONSES

Cell-mediated cytotoxicity is primarily responsible for allograft rejection, and this also appears to hold true for tumor rejection. Thymus-derived lymphocytes (T cells) have been shown to be capable of specific cytotoxicity following sensitization _in vivo_ to alloantigens (12,13). This killing is not known to require any accessory cells or factors (13) and thus corresponds well with the observations of Old, Boyse et al. (4) that immunity to chemically induced tumors could be transfered with cells. Similarly, Le Clerc et al. (14) have shown the presence of lytic T cells in a syngeneic model with a murine sarcoma virus tumor.

Controversy still exists over whether or not the lymphocytes of a tumor-bearing host are cytolytic _in vivo_ for the growing tumor. The Hellströms and their coworkers have published numerous reports supporting the existence of such cytolytic cells (29,31) while Mikulska, Smith and Alexander (17) find active cells only if animals are examined three weeks after surgical tumor excision; spleen cells collected while the tumor was still in place showed no cytolytic activity. The difficulty in comparing and evaluating conflicting reports on this subject stems from the variety of assay systems employed by various authors. The Hellströms have used the colony inhibition assay (CI) (18) and the microcytotoxicity test (MA) of Takasugi and Klein (19) both of which require extensive cell culture periods. This has left their work open to the charge that lymphocytes are sensitized to the tumor cells _in vitro_ but are

not sensitized in the tumor-bearing host. Mikulska et al. (17) used a neutralization assay in which spleen cells were mixed with tumor cells and re-injected into the experimental animals. Tumor cells mixed with spleen cells from an immune animal or an animal whose tumor was surgically removed 3 weeks earlier did not grow, while tumor cells mixed with spleen cells from a tumor-bearing animal did grow. In an attempt to resolve these difficulties Plata and Levy (20,21) have recently published a series of studies on the effector cells in the MA and chromium release test (CRT). Their data indicate a fundamental difference in the nature of the two tests. Microcytotoxicity assay requires a 48 hours incubation _in vitro_ and measures a cytostatic, rather than cytolytic activity; it involves both T and non-T effector cells; it is inhibited by serum from tumor-bearing animals; and it shows a biphasic response with an intermediate period of no reactivity which corresponds to maximum tumor size. Chromium release test requires an 18 hour incubation _in vitro_ and measures cytotoxic activity; it involves only T cells; it is not inhibited by serum from tumor-bearing animals; and it shows a monophasic response with maximum activity coinciding with maximum tumor size. Unfortunately, two factors make generalizations based on this data somewhat doubtful. In the first place the tumor chosen, a murine sarcoma virus induced tumor, regressed spontaneously starting approximately 15 days after injection and disappearing completely by day 25. All experimental subjects are thus capable of recognizing and rejecting the tumor, which may not be the case with progressively growing lethal tumors. Secondly it

was necessary to use two different target cell types for the two assays, neither of which were the tumor under study although all three carried the FMRGi antigen. The ascitic lymphoma used in CRT is non-adherent and hence not suitable for MA; the fibroblastic tumor cells used in MA could not be lysed by immune lymphocytes from MSV-tumor-bearing animals so were not suitable for CRT target cells.

However from this evidence, and some cell fractionation experiments using anti-Ig-coated glass bead columns or anti-θ treatment (20), it does seem clear that the two tests are measuring activities of separate cell populations. They conclude that MA detects T and non-T effector cells while CRT detects only effector T cells, but a different T cell population than that active in MA.

Cell-mediated cytotoxicity, effected by one or more T cell populations, thus is capable of destroying living tumor cells and can clearly effect rejection in an immune animal or upon transfer from an immune to a tumor-bearing animal. What is still unclear is the role played by these cells in the case of a progressively growing tumor which eventually destroys the host.

B. ANTIBODY-MEDIATED RESPONSES

(i) ANTIBODY

Cellular immunity, primarily T cell mediated cytotoxicity, has been regarded for some time as the principal anti-tumor defense while antibodies were regarded as ineffective or antagonistic, perhaps causing enhancement of tumor growth. Recent studies have shown this view to be an oversimplification.

Uhr and Möller (35) have defined immunological enhancement as "the prolonged survival of normal or neoplastic tissue grafts in histoincompatible recipients, which have been pretreated with antibodies directed against the graft or, alternatively, which have been presensitized with tissue of the graft genotype". Kaliss (22) showed that the active factor was circulating antibody directed against the incompatible antigens. By contrasting these results with those obtained for cell-mediated immunity it is readily apparent how the idea arose that immune lymphocytes, whether actively or passively induced, produced protection while specific antibody, again either actively or passively supplied, caused enhanced tumor growth. Initial studies were done with transplantation antigens, primarily H-2, but G. Möller (25) showed that anti-tumor antiserum could cause enhanced tumor growth *in vivo*. Gorer and Kaliss (23) had already shown that the result of passive administration of antiserum was not always enhancement, but depended rather upon the sensitivity of transplanted cells to lysis by antibody plus complement. However several papers by E. Möller (26-28) indicated that enhancement may have an important role in maintaining

autochthonous tumors and this led to the extensive studies by the Hellströms and their associates into the role of anti-tumor antibodies in tumor survival. In a review of their early work, Hellström and Hellström (30) describe a series of experiments done with mouse, rabbit, and human tumors in which it was shown that lymphocytes from subjects which had rejected a tumor were capable of significantly inhibiting tumor cell growth in the colony inhibition assay. Much to their surprise, however, lymphocytes from tumor-bearing subjects were equally effective. Prompted by the work on enhancement they examined the effect of serum from the various experimental subjects. Serum from normal subjects or those which had rejected the tumor showed no effect on either lymphocyte sample, however serum from subjects bearing a progressively growing tumor blocked the ability of lymphocytes to inhibit tumor cell colony growth. (29) This serum blocking factor showed specificity for tumor cell type, and could be removed by absorption with the corresponding tumor cells. Serum antibody was further implicated by the fact that activity could be removed by precipitation with anti-immunoglobulin antiserum, and that activity resides in the 7S fraction of serum.

Subsequent work indicated a more complex situation. Low pH eluates from human tumor cells (31), or fractions prepared by absorption and elution of tumor-bearing-mouse serum from corresponding tumor cells (32), could be separated into two fractions by ultrafiltration at low pH. If the fractions were added to the target cells for 45 minutes then removed, neither alone had any effect on cytotoxicity by sensitized lymphocytes while a 1:1 mixture

effectively blocked lymphocyte activity. This led them to propose that the blocking factor was an antigen-antibody complex rather than antibody alone.

Several facts could not be easily reconciled with this theory: blocking activity disappeared rapidly from serum following surgical removal of tumor, to be replaced by cytotoxic antibody (30); and the low-molecular weight eluate, but not the high molecular weight one, could block alone if allowed to remain in contact with lymphocytes and tumor cells for the duration of the assay.

An alternative explanation is that the active blocking factor is tumor antigen. According to this hypothesis, in the presence of an active immune response much of this antigen exists bound to serum antibody and consequently is localized in the 7S fraction of serum and can be bound to and eluted from tumor cells. Rapid disappearance of blocking activity following tumor removal would be due to loss of the source of antigen, antibody-bound antigen being rapidly removed from circulation leaving only cytotoxic antibodies whose production would continue for some time. Activity of the low pH eluates could be explained as follows: brief incubation of tumor cells with antibody or antibody-antigen complexes would allow binding and subsequent transfer to the microcytotoxicity assay where antigen from the complex blocks lymphocyte action but neither preincubation of tumor cells with antibody alone nor antigen alone would transfer antigen to the assay; if the 3 samples are added directly to the microcytotoxicity assay antigen alone or antigen-antibody complexes should both produce blocking, as was observed.

Arguments of this type have made "blocking factor" an unpopular

topic, despite widespread initial enthusiasm for the concept. It would be wrong however to assume that if blocking is due to antigen released by tumor cells, anti-tumor antibody is unimportant to the host response. Specific graft enhancement by antibody is well-documented; and Vánky et al. (33) have shown that preincubation of sarcoma cells with autochthonous serum completely abolished their ability to stimulate autochthonous lymphocytes as measured by uptake of radioactive thymidine. Even if antibody is shown not to be relevant to the blocking observed by the Hellströms, antibody or antibody-antigen complexes may have an important role in regulation of the immune response, a topic which is discussed in a later section.

The Hellströms also reported that following regression of a tumor cytotoxic antibodies could be detected in the circulation (30). Old, Boyse and Lilly (37) made similar observations in a mouse tumor system. Studying strains of mice normally susceptible to tumor induction by Friend virus they observed occasional tumor rejection. Serum from these mice could be shown to be cytotoxic for tumor cells _in vitro_ in the presence of complement. _In vitro_ cytotoxicity does not always correlate with protection _in vivo_, however in another study (38) they showed that an antiserum specific for the G antigen could be used to passively immunize mice against challenge with a syngeneic Gross tumor.

Herberman and Oren (36) also studied a Gross virus induced tumor in W/Fu rats. This tumor is strongly antigenic and regressed in 65 percent of the recipients within two weeks. In the other rats the tumor grew progressively or regressed after an extended period of

time. All rats produced specific, highly cytotoxic antibodies but these did not correlate with protection *in vivo*. The responses all showed a biphasic response in which the first peak could be shown to be 19S antibodies and the second peak 7S antibodies. The titre and duration of the secondary response was proportional to the duration of tumor growth, the highest titre being present in rats which died shortly afterward. It would appear that in this case cytotoxic antibody was produced in response to continued antigenic stimulus but conferred no protection on the host animal.

Several explanations have been offered to account for the apparent ineffectiveness of such antiserum which is so strongly cytotoxic *in vitro* yet provides no protection *in vivo*. Gorer and Kaliss (23) noted in an early study on enhancement of murine sarcoma that the ultimate effect often depended upon the amount of antiserum given; while small doses enhanced tumor growth, larger doses could be cytotoxic. Similar results were obtained by Möller (39) with leukemia cells. The resistance or susceptibility of cells to lysis by antibody plus complement is also a factor to be considered. Growth of cells highly resistant to lysis by antibody due to decreased surface antigen (40) would presumably be more readily enhanced, while more susceptible cells would be destroyed by the presence of the same antiserum.

Results obtained in the AKR strain of mice, which is highly susceptible to leukemia, point up the importance of factors external to the tumor cell — antibody system. It was found that infusion of normal serum into AKR mice bearing spontaneous leukemias caused rapid destruction of leukemia cells. Evidence points to a complement

component deficiency as being responsible, in this case probably C5 (41). The authors also refer to preliminary experiments which indicate that complement may be the limiting factor in determining the effectiveness of antibody-mediated tumor cell destruction in the mouse.

These explanations may help to account for the ineffectiveness of antibody is some situations but they do not exclude the possibility that cytotoxic antibody has a role in tumor cell destruction, especially with susceptible cells such as leukemias (40), in situations where the accessory systems, such as complement, are adequate.