AD________________

Award Number: DAMD17-00-1-0359

TITLE: Dissecting the Immunogenicity of Monoclonal Antibodies

PRINCIPAL INVESTIGATOR: Christopher M. Snyder
Lawrence J. Wysocki

CONTRACTING ORGANIZATION: The National Jewish Medical and Research Center
Denver, Colorado 80206

REPORT DATE: June 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20011128 207

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2001	Annual (1 Jun 00 - 31 May 01)

4. TITLE AND SUBTITLE
Dissecting the Immunogenicity of Monoclonal Antibodies

5. FUNDING NUMBERS
DAMD17-00-1-0359

6. AUTHOR(S)
Christopher M. Snyder
Lawrence J. Wysocki

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The National Jewish Medical
and Research Center
Denver, Colorado 80206
E-Mail: christopher.snyder@uchsc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The potential of mononclonal antibodies, (mAbs), for use in therapeutic and diagnostic applications has not been fully realized in part due to counter-immune responses that often arise in patient recipients of mAb. A growing research effort to "humanize" mAb has focused primarily on the structure or sequence of the antibody variable (V) region domains. However, these approaches may ultimately suffer, as they overlook the requirement of T cell help for the immune counter-reaction and the potential of somatic hypermutation and V-D-J recombination to generate target T cell epitopes within mAb V regions. My approach focuses on this issue. In order to understand some basic principals concerning anti-immunoglobulin immune responses, I have developed methods, reagents and mouse strains during the previous funding year to effectively study T cell epitopes in mAb V regions. I was able to reproducibly distinguish an immune response to a V-region T cell epitope via a lymph node proliferation assay. Furthermore, I have generated MHC-tetrameric staining reagents with which I can identify T cells reactive with two such epitopes. Finally, I have generated congenic mouse strains containing or lacking the enzyme terminal deoxynucleotidyl transferase, (TdT), in order to address the role of T cell epitopes in junctional diversity.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award) Tumor Therapy and Detection, Immunology
Monoclonal Antibodies, Counter-Immune Response

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover 1

SF 298 2

Table of Contents 3

Introduction 4

Body 5

Key Research 9

Reportable Outcomes .... None

Conclusions 10

References 11

Introduction:

Monoclonal antibodies have been increasingly used for therapeutic and diagnostic applications. Their potential use in targeting tumor antigens, specifically those expressed by carcinomas of the breast, is just now beginning to be appreciated. Successful application of monoclonal antibodies has been constrained by counter-immune responses elicited in patient recipients. Counter-immune responses can limit the duration and number of times that a patient can receive the therapy and may result in potentially dangerous reactions. Efforts to avoid this response have mostly centered on a variety of strategies to "humanize" monoclonal antibodies. The rationale behind this approach is that "humanized" antibodies will have few or no epitopes which could be viewed as "foreign" by the recipient's immune system. However, mounting an immune response to a protein antigen generally requires both B and T lymphocytes, and a memory response always requires both cell types. In general, current efforts to humanize monoclonal antibodies have overlooked potential T cell epitopes present within the mAb variable region.

Previous data from several laboratories, including my own, have suggested that T cells can respond to epitopes created in immunoglobulin variable regions through the physiological somatic hypermutation process [1, 2]. Furthermore, it has been proposed that the generation of junctional diversity by V-D-J gene recombination can produce T cell epitopes. However, to my knowledge, this has not been extensively studied and incorporated as a humanization strategy. My view is that the oversight of these naturally occurring T cell epitopes is a major drawback to current humanization approaches. Consequently, this proposal is aimed at understanding the prevalence of T cell epitopes in monoclonal antibodies generated naturally by both somatic hypermutation and junctional diversification of the third complementarity determining region (CDR3).

Body:

In aim #1 of my proposal, I outlined a plan to assess the immunogenicity of an antibody with a germline-encoded T cell epitope in highly defined syngeneic mice. To test this question, I proposed to immunize congenic strains of mice with a well defined germline-encoded T cell epitope located in the variable region of monoclonal antibody, (mAb), 36-65. The natural V-gene encoding this epitope has been bred onto the C58 background providing me with congenic C58 strains which either contain or lack the gene. These experiments aim to test the idea that germline encoded antibody sequences may be non-immunogenic while somatically generated epitopes are immunogenic even in syngeneic animals. Thus, if my hypothesis is correct, simply removing any somatically generated epitopes from a mAb may reduce its immunogenicity significantly. Previously, I addressed this question by making T cell hybridomas. However, this technique is arduous and time consuming. In order to analyze any significant number of mice I proposed to develop alternative methods to assess immunogenicity. Therefore, most progress on this aim has centered on the development of sensitive techniques to measure T cell responses to immunoglobulin peptides without having to generate T cell hybridomas.

I have pursued two different methods to analyze *ex vivo* immunoglobulin-reactive T cells. First, I chose to develop a lymph node proliferation assay sensitive enough to detect T cell responses to antibody variable regions. While lymph node proliferation assays have been used successfully to study normal T cell responses to foreign antigens, the small number of T cells specific for syngeneic immunoglobulin peptides has hindered this approach for my purposes. In order to more easily develop this technique, I chose a well defined T cell response in which T cells from A/J mice recognize and respond to an epitope in the variable region of mAb 36-71. The epitope was created by somatic hypermutation, which produced two amino acid replacements in the kappa V region. Interestingly, these mutations created an MHC binding peptide where none existed before. Consequently, this is a good model to begin addressing the effect of T cell epitopes in syngeneic mAbs, as similar mutations may occur in "humanized" mAbs produced in mice that carry human immunoglobulin transgenes.

My early attempts to develop a lymph node proliferation assay were stifled by a high level of background proliferation that occurred in the absence of deliberately added antigen. Attempts were made to reduce this background by changing the duration, route and delivery of the immunizations. Further attempts to reconcile the issues with background were made by testing irradiated vs. un-irradiated antigen presenting cells, (APCs). Again however, no specific response could be detected above background. Promising results were obtained when T cells were purified via nylon wool columns. However, the low numbers of T cells recovered by this technique proved to be a major restriction. Ultimately, I began using magnetic separation of T cells, (Stem Cell Technologies-Vancouver, Canada). This technique allowed me to purify much larger numbers of T cells from individual mice. I have now successfully developed a protocol through which specific responses can be detected, (Figure 1). Shown in Figure 1A is a control lymph node proliferation response to ovalbumin. In Figure 1B, mice were immunized with a peptide representing the mutated epitope in mAb 36-71. Using this technique, the critical experiments outlined in Aim #1 of my proposal can now be undertaken in a more expiditious manner.

Concurrent with this approach, I pursued the development of class II MHC tetramers. This relatively new technology, is useful in analyzing T cell responses as is increasingly evident in recent literature [3, 4]. Although this approach was not outlined in my original aims tetramers can be used to address the original question. These staining reagents should allow us to visualize specific T cells participating in the immune response to immunoglobulin peptides. Two such tetramers have been constructed. The first tetramer consists of an I-A^k MHC and contains the mutant epitope from the kappa V region of mAb 36-71. Initially, a construct was created containing 11 amino acids from mAb 36-71. This construct did not yield any detectable protein when expressed in a baculovirus expression system. Subsequent constructs were made containing 13 amino

acids from the same sequence of mAb 36-71 with the hope that increased peptide flexibility would allow for more stable complexes. However, this also did not yield any protein. I speculated that the stability of the recombinant I-A^k molecules was very poor. In order to overcome this difficulty, I added a leucine zipper to the C-terminal end of both the alpha and beta chains of the protein. The leucine zipper provided stability for the alpha/beta MHC dimer and productive protein was expressed, (Figure 2A). The sequence of the peptide is shown in Figure 2B.

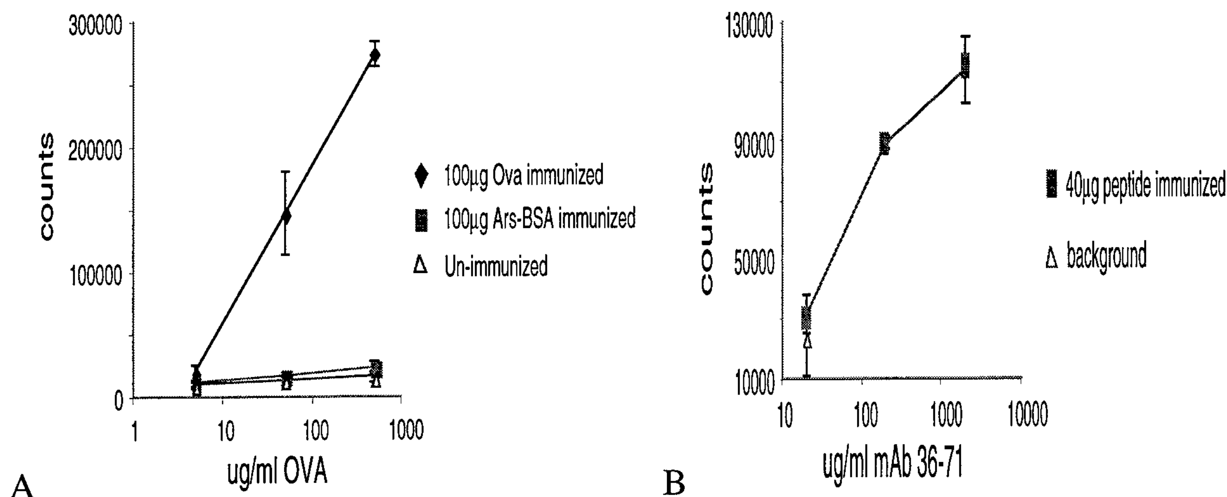
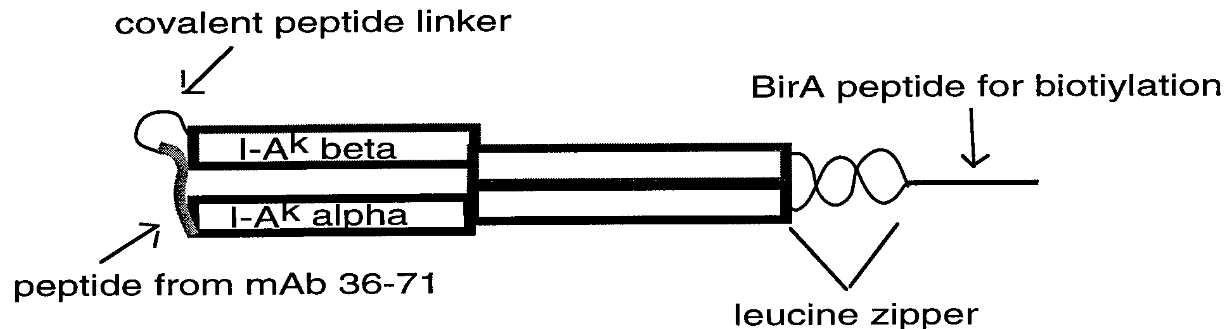


Figure 1: Examples of lymph node proliferation assays. The assay was performed by immunizing mice sub-cutaneously in the flank with the indicated amount of antigen. Draining inguinal and peri-aortic lymph nodes were harvested 14 days later from individual mice. T cells were purified from the lymph nodes using magnetic separation, (Stem Cell Technologies). 200,000 T cells and 400,000 splenocytes from an unimmunized mouse were plated with the indicated antigen concentrations. The background shown in figure B is derived from wells with no antigen added.

Figure 2
A:



B:

D I Q M T Q I P S S L S A----covalent peptide linker to I-A^k-beta

Figure 2: Figure A shows a schematic of the I-A^k tetrameric construct including the leucine zipper ultimately used for protein stability. Figure B shows the 13 amino acid peptide sequence derived from mAb 36-71. The underlined isoleucine and proline are the amino acids created by somatic hypermutation.

Preliminary data suggested that this tetramer was specific for 36-71 reactive T cell hybridomas. However, a true demonstration of its usefulness could only be achieved by staining *ex vivo* T lymphocytes. To this end, I immunized mice with mAb 36-71 or peptide containing the 13 amino acids used to make the tetramer. An example of the staining data is shown in Figure 3. To date, 7 of 7 mice, immunized with either mAb 36-71 or

corresponding peptide have shown a significant expansion of tetramer staining CD4 positive T cells either 7 or 14 days after immunization. Other mice have been immunized with mAb 36-65 as a control. This mAb is identical to mAb 36-71 except that it lacks any somatic hypermutations. Results similar to the negative control shown in Figure 3 have been seen for these mice.

Figure 3

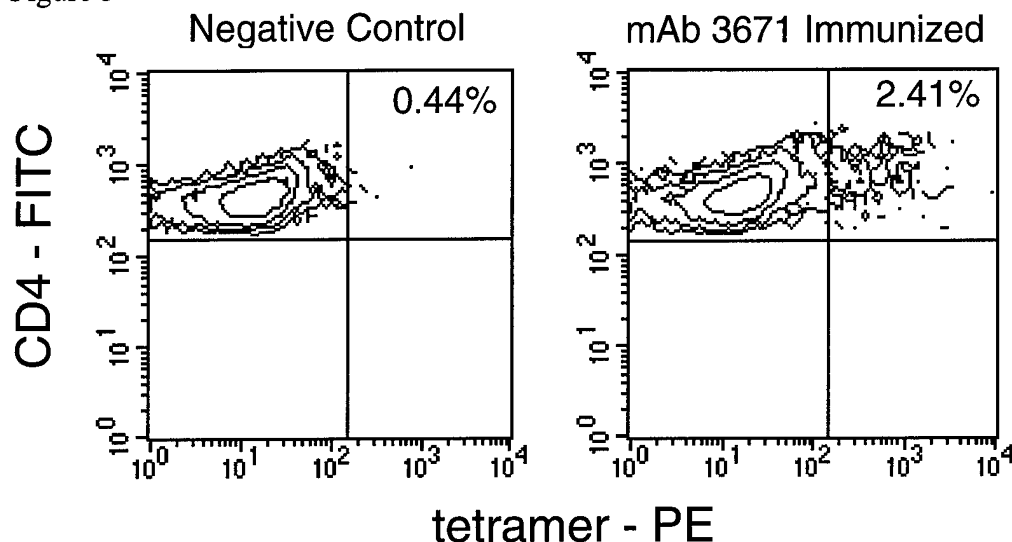


Figure 3: Mice were immunized sub-cutaneously with 100μg 3671 mAb or left unimmunized. Draining inguinal and peri-aortic lymph nodes were harvested 14 days later and stained with IAk-3671 tetramer reagent. The plots shown were first gated on B220 negative, CD4 positive and CD44-hi, live lymphocytes. Tetramer positive cells appear in the upper right quadrant. The percentages shown are of the gated population.

Further efforts to create an I-E^k tetramer containing the germline immunoglobulin epitope from mAb 36-65 are proceeding smoothly. I have evidence that my construct makes productive protein in the baculovirus system without the need for a leucine zipper. However, no other preliminary data is available regarding this tetramer. This staining reagent will allow me to study the T cell response in the congenic C58 strains either containing or lacking the germline V-gene mentioned above.

The tetramers and lymph node proliferation assays have given us the ability to study the T cell responses to a somatically-generated or a germline-encoded immunoglobulin epitope in a large number of individual mice. Thus, future experiments that these tools allow promise to lead to fundamental observations regarding the nature of T cell responses to immunoglobulin diversity. My working hypothesis is that mice attain a state of tolerance to germline-encoded sequences, but that somatically-generated sequences may be immunogenic.

Aim #2 of my proposal addressed the question of junctional diversity. This aim centered on immunizing mice with unmutated monoclonal antibodies and developing T cell hybridomas against any immunogenic mAbs. To this end, 8 different mAb containing known sequences and various junctions have been purified and are ready for immunization. Further, progress on this aim was postponed, pending the development of the lymph node proliferation assay.

Aim #3 of my proposal addresses the hypothesis that junctional diversity in CDR3 may be a significant source of T cell epitopes in mAbs. This aim tests the immunogenicity of junctional diversity on a much more global scale than Aim #2 by using mice that either express or lack the enzyme terminal deoxynucleotidyl transferase, or TdT. Mice lacking TdT contain very limited junctional diversity in CDR3. This aim is important to the humanization effort, because human antibodies made in mice with human transgenes could contain CDR3 epitopes that serve as potential avenues of T cell help for a counter-immune response in human recipients of such mAb. Thus, removing TdT from the animal used to make a mAb may reduce or eliminate a potential

counter-immune response in a recipient (patient). In order to address this question, I proposed to study the immunogenicity of mAbs produced in TdT proficient mice. To this end, I have completed the generation of congenic mice on the SWR background that either contain or lack the enzyme TdT. In theory T cells from these mice should be capable of responding to any T cell epitopes in immunoglobulin created by the full diversity present in TdT proficient mice. Therefore, I am currently conducting preliminary experiments to address the ability of T cells from one strain to proliferate in response to epitopes presented by the opposite strain using the fluorescent dyes CFSE (green) and PKH-26 (red). Fluorescent cell trackers have been used extensively in the literature to follow proliferation in various cell populations [5, 6]. In this case, I have been using the dyes to track the proliferation of T cells which respond to junctional diversity *in vivo*. To date, I have established adoptive transfer protocols and am conducting preliminary experiments. The experiments outlined in aim #3 of my proposal will be undertaken to further address the frequency of any junctional T cell epitopes in these congenic mouse strains.

Key Research Accomplishments

- development of a sensitive lymph node proliferation assay which can measure T cell responses to epitopes found in immunoglobulin variable regions

- development of tetrameric reagents that can recognize T cells specific for both mutated and germline epitopes identified from immunoglobulin variable regions.

- Purification and sequencing of 8 mAbs containing varying V-D-J junctions

- generation of congenic mouse strains containing and lacking TdT.

- development of adoptive transfer and staining protocols to track the proliferation of T cells *in vivo*.

Conclusions

The research completed to this point is important for my ability to move forward and address the proposed questions. Much time has been devoted to establishing reproducible and reliable techniques with which to study T cell epitopes created by somatic hypermutation and junctional diversification. Ultimately, this research should help to elucidate the significance and prevalence of T cell epitopes found within mAb variable regions. With this knowledge it may be possible to design more effective means of producing mAbs so that T cell epitopes are minimized. For example, humanizing mAbs from TdT deficient mice may prove essential for the elimination of T cell epitopes from junctional regions. This research should contribute to the expanding body of knowledge relating to the counter-immune response against monoclonal antibody therapies.

References

1. Bogen, B., T. Jorgensen, and K. Hannestad, *T helper cell recognition of idiotopes on lambda 2 light chains of M315 and T952: evidence for dependence on somatic mutations in the third hypervariable region.* Eur J Immunol, 1985. **15**(3): p. 278-81.
2. Eyerman, M.C. and L. Wysocki, *T cell recognition of somatically-generated Ab diversity.* J Immunol, 1994. **152**(4): p. 1569-77.
3. Rees, W., et al., *An inverse relationship beween T cell receptor affinity and antigen dose during CD4(+) T cell responses in vivo and in vitro.* Proc Natl Acad Sci U S A, 1999. **96**(17): p. 9781-6.
4. Savage, P.A., J.J. Boniface, and M.M. Davis, *A kinetic basis for T cell receptor repertoire selection during an immune response.* Immunity, 1999. **10**(4): p. 485-92.
5. Bender, J., et al., *CD4+ T cell division in irradiated mice requires peptides distinct from those responsible for thymic selection.* J Exp Med, 1999. **190**(3): p. 367-74.
6. Boutonnat, J., et al., *PKH26 probe in the study of the proliferation of chemoresistant leukemic sublines.* Anticancer Res, 1998. **18**(6A): p. 4243-51.