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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 devloped a panal of T cell hybridomas and new transgenic mice. Studies with these tools strongly suppport our basic hypothesis that T cells are tolerant of endogenous immunoglobulin-derived diversity. I have also obtained a panal of T cell hybriomas that are specific for the CDR3 region of a monoclonal antibody supporting our hypthesis that junctional diversity may provide a source of T cell epitopes within a monoclonal antibody. Finally, I have addressed the global nature of T cell responses to junctional diversity with an adoptove transfer system.

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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 that 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 ours, 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 that 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.

I originally pursued the development of several techniques with which 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. The majority of the progress with this technique was described in last year's annual report. While this assay seemed very promising from the preliminary data obtained in A/J mice, repeated attempts to detect proliferative responses above background levels in C58 mice have failed. Recent data has suggested that the smaller lymph node responses may be linked to the immunization protocol itself. Studies are currently being performed to address this question in more detail

Concurrent with this approach, I have also 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 effectively 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, which was described in last year's annual report, is an IA^k-MHC construct containing a mutant peptide obtained from the V-region of the monoclonal antibody 36-71. The second tetramer is an $I\text{-}E^k$-MHC construct containing the germline immunoglobulin epitope from mAb 36-65. While I have successfully developed both of these constructs, technical difficulties in the areas of protein expression and purification have been a major stumbling block in their use. Once these difficulties are solved, these staining reagents will allow me to study the T cell responses to immunoglobulin-derived peptides by flow cytometry.

I have also pursued the analysis of T cell hybridomas derived from our congenic C58 strains. While arduous, this technique has been well worked out by our laboratory and has been successfully used for analyzing the T cell responses of C58 mice in the past [5]. For this analysis, either our C58 mice or our congenic C58 mice containing the germline gene used by mAb 36-65 are immunized with a peptide encoded by the germline gene in question. Lymph nodes from a recently, (3-4 days), boosted animal are then harvested and fused *in vitro*. Individual hybridomas resulting from the fusion are then screened for reactivity to both the immunizing peptide and the parent antibody. Over 300 T cell hybridomas have been obtained from immunizations of these congenic strains of mice. The screening process is currently underway and 101 of the hybridomas have been fully tested for immunoglobulin-specific reactivity. Thus far, the data confirms our previous findings indicating that C58 mice, which lack the variable gene, are indeed able to respond to epitopes encoded by this gene. Conversely, none of the tested hybridomas derived from congenic C58 mice, which express the variable gene, were found have any immunoglobulin specific reactivity. As more cells are screened, more definitive conclusions may be drawn from this research. However, the evidence obtained thus far confirms our hypothesis stating that T cells will be tolerant of their own immunoglobulin diversity.

The techniques described to this point are all indirect measurements of tolerance to a variable region peptide. Therefore, in an effort to more fully understand and define the nature of a T cell response to endogenous immunoglobulin diversity I have developed a T cell receptor (TCR) transgenic mouse. The mice have been designated A30 after the hybridoma from which the TCR genes were cloned. The T cells from the A30 mice

are specific for a peptide derived from the mAb 36-71 that contains naturally derived somatic mutations, which created a T cell epitope. The large number of immunoglubulin specific T cells in the TCR transgenic mice allows me to directly observe the development of tolerance to a variable region peptide. Previous work in the laboratory led to the construction of an immunoglobulin transgenic mouse that expresses the mutated kappa-light chain from mAb 36-71. Thus, B cells from the Vκ 36-71 transgenic mouse express the epitope recognized specifically by the T cells from the A30 transgenic mouse. Even though these particular mice were not discussed in my original proposal, they provide me with a more dynamic and physiological experimental model to address the specific question described in Aim #1. To this end, we generated mice expressing both the TCR and kappa-light chain transgenes, which have allowed me to investigate the phenotype of a T cell that is specific for an endogenous immunoglobulin variable-region peptide. As such, significant time and effort has been spent on the analysis of these mice.

T cells from the A30 transgenic mice express the transgenic receptor at a very high frequency (~98%), (Figure 1A). Furthermore, these T cells respond vigorously both *in vitro* and *in vivo* to the appropriate peptide known as peptide #894, Figure (1B, and 1C). Furthermore, the transgenic T cells also respond to the epitope endogenously generated by transgenic B cells, (Figure 2). Serial dilution experiments have indicated that virtually all of the T cells in A30 transgenic lymph nodes are specific for the peptide derived from Vκ 36-71 transgenic mice, (not shown). This result is confirmed by analyzing the double transgenic offspring of A30 transgenic and Vκ 36-71 transgenic mice. As predicted, virtually all (>97%) of the CD4+ T cells are deleted in the thymus in mice expressing both the T cells and the specific epitope, (Figure 3A and B).

Figure 1

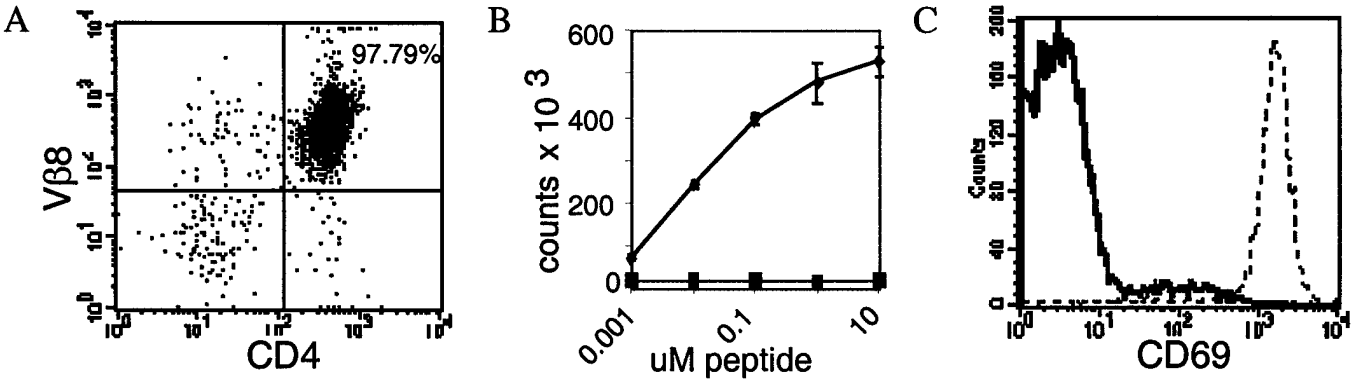

Figure 1: A) FACS analysis of a TCR transgenic A30 mouse. The cells shown were gated on the CD3 positive population first. B) Proliferation of A30 Tg cells (blue line) or non-transgenic littermates (pink line) *in vitro* was measured by H^3 incorporation after 2 days of culture with the specific peptide #894. C) FACS analysis of A30 Tg cells that were injected i.v. 20 hours previously with 5μg of the specific peptide #894 (dotted line) or with PBS alone (solid line). Greater than 98% of the CD4+ cells from peptide injected mice were CD69 high after 20 hours.

These data support our general hypothesis stating that mice will be tolerant of endogenous germline encoded epitopes derived from immunoglobulin variable regions, but may be reactive towards diversity generated through somatic mutation. In our double transgenic model, T cells which recognize an endogenous immunoglobulin-derived epitope are deleted in the thymus suggesting that this may be the mechanism of T cell tolerance to variable region diversity. However, if the TCR transgenic mice lack the 36-71 derived epitope, the T cells are fully responsive both *in vitro* and *in vivo* to various methods of stimulation. These observations would not have been possible without the high resolution studies allowed by the transgenic system. Through the analysis of our T cell hybridomas as well as further experimentation with our TCR transgenic mouse, I hope to understand the nature of this tolerance in greater detail during the coming year. The tetramers and lymph node proliferation assays will ultimately give us the ability to study both the transgenic and endogenous T cell responses to a somatically-generated or a germline-encoded immunoglobulin epitope in a large number of

individual mice. Thus, ongoing and future experiments promise to lead to fundamental observations regarding the nature of T cell responses to immunoglobulin diversity.

Figure 2

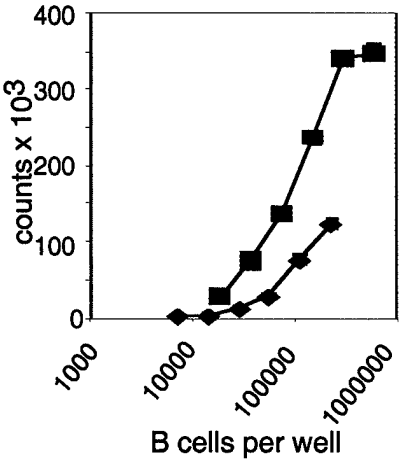

Figure 2: A30 transgenic T cells respond *in vitro* to splenocytes expressing the 36-71 light chain. A30 Tg lymph node cells were incubated for 2 days with splenocytes from a Vκ 36-71 transgenic mouse fractionated by density through a Percoll column. After 24 hours, the wells were pulsed with H^3 and incubated for a final 24 hours. A fixed number of A30 LN cells (100,000 per well) were incubated with varying numbers of the APC. The number of B cells per well is based on the fraction of cells expressing B220 as seen by FACS analysis. Low density splenocytes, which are traditionally thought to be more activated, are shown in pink. High density cells, which are traditionally thought to be more resting, are shown in blue.

Figure 3

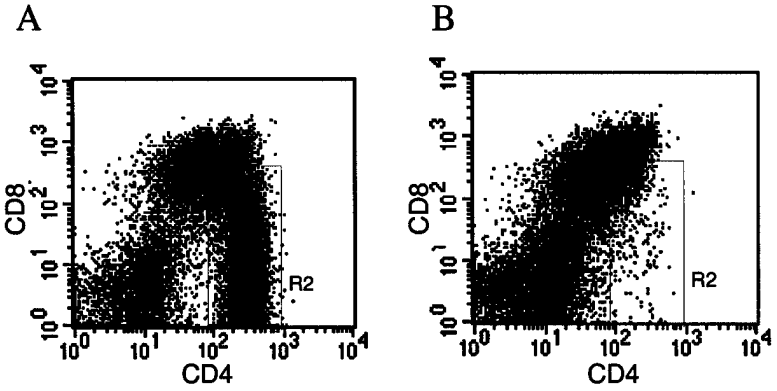

Figure 3: A30 transgenic T cells are deleted in the thymus of mice expressing the 36-71 light chain. A) FACS analysis of A30 Tg thymocytes from a 3 week old mouse. B) FACS analysis of thymocytes from a 3 week old (A30 x Vκ 36-71)F1 double transgenic mouse.

Aim #2 of my proposal addressed the question of whether immunoglobulin junctional diversity was immunogenic with respect to T cells. This aim centered on immunizing mice with unmutated monoclonal antibodies and testing for T cell responses to directed against the third complementarity determining regions (CDR3) of the respective heavy and light chains. However, technical difficulties in developing a consistent lymph node proliferation assay have necessitated that I assay for immunogenicity by an alternative method. For this reason, I am focusing my efforts on 7 interesting and potentially informative T cell hybridomas derived from a mouse that was immunized with a monoclonal antibody. Preliminary results indicate that all 7 of the T cell hybridomas respond to a pair of synthetic peptides spanning the portion of the heavy chain CDR3 formed at the junctional boundary of the variable (V) and diversity (D) gene segments. Thus, these hybridomas provide me with a direct test of the hypothesis put forth in aim #2 of my proposal. In the next year I plan to confirm this result and show whether the hybridomas are restricted by class II MHC molecules. This should provide a formal demonstration that CDR3 junctional diversity is indeed immunogenic for class II MHC-restricted helper T cells.

Aim #3 of my proposal is an extension of Aim#2 in that it tests more globally the hypothesis that junctional diversity in CDR3 may be a significant source of T cell epitopes in mAbs. The strategy is to make use of mice that either express or lack the enzyme terminal deoxynucleotidyl transferase, or TdT. Mice lacking TdT contain extremely limited junctional diversity within the antibody CDR3. If CDR3 frequently contain immunogenic

epitopes for T cells in normal mice, then antibodies from normal (TdT-wild type) mice should be immunogenic with respect to T cells in TdT-deficient mice. This aim is important to the humanization effort, because human antibodies made in mice that carry 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-wild type 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 antibody CDR3 epitopes created by TdT in wild type mice. Therefore, I have conducted experiments to address the ability of T cells from the TdT-deficient to proliferate in response to epitopes presented by the TdT-wild type 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 [6, 7]. In this case, I labeled T cells with the dyes to track the proliferation of T cells that responded to TdT-derived CDR3 diversity *in vivo*. The results of my experiments revealed no obvious global proliferation within the T cell compartment at the various time points tested. Therefore, it may be rare for a T cell to be specific for CDR3 junctionally diversity, or conversely, it may be very rare for junctional diversity to generate a T cell epitope. However, the preliminary results from Aim #2 would indicate that some CDR3 junctional diversity is immunogenic. Thus, if these data can be confirmed, it would suggest that the strategy of making anti-tumor mAb in TdT-deficient mice that carry transgenes encoding human immunoglobulin is a worthwhile strategy that could lead to benefits in the clinic.

Key Research Accomplishments

- Development of a panel of T cell hybridomas from both of the congenic C58 strains of mice.

- Screening of roughly 1/3 of the T cell hybridoma panel generated from the congenic C58 strains. These data support the hypothesis that mice are tolerant of germline encoded antibody diversity.

- Development of the TCR transgenic mice designated A30 with which high resolution analysis of T cell responses to immunoglobulin-derived epitopes can be observed.

- Analysis of double transgenic mice expressing both the A30 T cells and the Vκ 36-71 transgenic B cells. This analysis confirmed our hypothesis that mice are tolerant of germline encoded diversity by revealing the deletion of specific T cells.

-Preliminary evidence that the heavy chain CDR3 of one mAb contains a T cell epitope.

- Analysis of the proliferative responses of T cells from congenic TdT-deficient animals adoptively transferred into TdT proficient animals. These studies suggested that few T cells from TdT-deficient animals are specific for epitopes generated by junctional diversity.

Reportable Outcomes

- Presentation of an abstract

Title: "Class II MHC-Restricted Presentation of BCR-Associated Epitopes Dependent Upon B Cell Activation".

Authors: Christopher M. Snyder, Xianghua Zhang, Lawrence J. Wysocki

Presented by: Christopher M. Snyder

- Publication of a manuscript in the Journal of Immunology.

"Negligible Class II MHC Presentation of BCR-Derived Peptides by High-Density, Resting B Cells"

Christopher M. Snyder, Xianghua Zhang, Lawrence J. Wysocki

J. Immuol. 2002 168: 3865-3873

- Production of novel T cell receptor transgenic mouse known as A30.

Conclusions

The research completed to this point is important in that it allows us to clearly define the observed tolerance of T cells to endogenously encoded immunoglobulin epitopes. Careful analysis of the hybridomas generated in the congenic C58 mice will allow us to clearly address the existence and/or role of T cell responses to endogenous immunoglobulin epitopes. Furthermore, analysis of TCR transgenic cells and their responses to the immunoglobulin-derived peptide in various scenarios should elucidate how T cells might contribute to the counter immune response elicited by monoclonal antibodies. This research should ultimately contribute to the expanding body of knowledge relating to the counter-immune response against monoclonal antibody therapies.

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