CHARACTERIZATION OF A T LYMPHOCYTE-DERIVED, ANTIGEN-BINDING MOLECULE WITH SUPPRESSIVE ACTIVITY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in
THE FACULTY OF GRADUATE STUDIES
Microbiology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
NOVEMBER 1989
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Date **Dec 20/89**
Regulation of the immune response is mediated, in part, by the action of suppressor T cells (Ts). One intriguing aspect of these cells is the description of T cell suppressor factor (TsF): a soluble analog of the cell that shares many of its properties, such as the ability to bind free antigen (Ag) and suppress an Ag-specific immune response. The exact molecular nature of TsF and the relationship of TsF to Ts are unknown. The immune response to the small, bacterial protein, ferredoxin (Fd), was used as a model system to study TsF. A Fd-specific suppressor cell network has been described in mice that are genetically nonresponsive to this Ag. Previously, a soluble mediator, known as Fdl1F, was found in the culture supernatant (SN) of the Ts hybridoma, Fdl1. Fdl1F possessed both Ag-binding activity and the ability to suppress the anti-Fd Ab response in mice. The TsF-specific monoclonal antibody, B16G, was used for both the recovery of Fdl1F-enriched material from SN and its detection by the enzyme-linked immunosorbent assay. Further immunochemical, biological, and biochemical characterization of Fdl1F was done with emphasis on describing the Ag-binding properties of Fdl1F. It was found that Fdl1F bound to solid- and liquid-phase Fd, and demonstrated preferential binding to the carrier determinant of the Ag. A spleen cell culture assay was devised which showed that Fdl1F suppressed Ab production in a concentration-dependent manner. Additional experiments suggested that the suppressive effect was
Ag-specific. The identification of the Ag-binding molecule was attempted by the fractionation of Fdl1F-enriched material using high performance gel filtration or preparative SDS-PAGE (run under non-reducing conditions). Using SDS-PAGE, a unique, single polypeptide of about 30k relative molecular mass ($M_r$) was identified as the Ag-binding moiety of Fdl1F. The possible relationship of this moiety to other identified materials is discussed.
TABLE OF CONTENTS

ABSTRACT ................................................................. ii
TABLE OF CONTENTS ....................................................... iv
LIST OF TABLES .......................................................... vii
LIST OF FIGURES ........................................................ viii
ACKNOWLEDGEMENTS ..................................................... x
ABBREVIATIONS ........................................................... xi

INTRODUCTION

Suppression and the Immune Response .............................. 1
Properties of T cell Suppressor Factors ............................. 2
  Summary .................................................................. 11
The Immune Response to Ferredoxin as a Model for the Study of
  Mechanisms of Immunoregulation .................................... 13
  The choice of Fd as a model antigen ................................. 14
  Epitope mapping and H-2 linked responsiveness to Fd ......... 15
  Summary .................................................................. 18
  Idiotype-anti-idiotype interactions .................................. 19
  Summary .................................................................. 22
B16G and the Development of the Fd11 hybridoma ............... 23
  Summary .................................................................. 27
Purpose of the Investigation ............................................. 28

MATERIALS AND METHODS

Buffers and Solutions ...................................................... 29
Experimental Animals ..................................................... 29
Cell Lines .................................................................. 29
Conventional and Monoclonal Antibodies ........................... 30
Antigens .................................................................. 32
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme-linked Immunosorbent Assay</td>
<td>33</td>
</tr>
<tr>
<td>Immunization of Animals</td>
<td>35</td>
</tr>
<tr>
<td>Enrichment of Fd11F using Immunoaffinity Chromatography</td>
<td>35</td>
</tr>
<tr>
<td>In vitro Measurement of Fd11F Suppressive Activity</td>
<td>37</td>
</tr>
<tr>
<td>Suppression of the Serum Antibody Response using Fd11F</td>
<td>39</td>
</tr>
<tr>
<td>Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
<td>40</td>
</tr>
<tr>
<td>High Performance Gel Filtration</td>
<td>41</td>
</tr>
<tr>
<td>Preparative Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
<td>42</td>
</tr>
<tr>
<td>Two-dimensional Gel Electrophoresis (IEF x SDS-PAGE)</td>
<td>43</td>
</tr>
<tr>
<td>RESULTS</td>
<td></td>
</tr>
<tr>
<td>Recovery of Fd11F from Culture Supernatants</td>
<td>45</td>
</tr>
<tr>
<td>Immunochemical Properties of Fd11F</td>
<td>45</td>
</tr>
<tr>
<td>Biological Properties of Fd11F</td>
<td>57</td>
</tr>
<tr>
<td>Development of an antibody culture assay for the measurement of suppression</td>
<td>57</td>
</tr>
<tr>
<td>Correlation between bioactivity and antigen-binding activity of Fd11F</td>
<td>61</td>
</tr>
<tr>
<td>Suppression of anti-Fd antibody levels in vivo</td>
<td>69</td>
</tr>
<tr>
<td>Biochemical Analysis of Fd11F</td>
<td>74</td>
</tr>
<tr>
<td>Introduction</td>
<td>74</td>
</tr>
<tr>
<td>Analysis of Fd11F using high performance gel filtration: correlation between antigen-binding and suppressive activity</td>
<td>75</td>
</tr>
<tr>
<td>Identification of the antigen-binding molecule using SDS-PAGE</td>
<td>81</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td></td>
</tr>
<tr>
<td>B16G as a Probe for T Suppressor Factor</td>
<td>91</td>
</tr>
<tr>
<td>Immunochemical Properties of Fd11F</td>
<td>93</td>
</tr>
<tr>
<td>Biologic Properties of Fd11F</td>
<td>96</td>
</tr>
</tbody>
</table>
Biochemical Analysis of Fd11F .......................... 101
Summary ................................................... 106
LITERATURE CITED ......................................... 108
APPENDIX  THE COMPOSITIONS OF BUFFERS AND SOLUTIONS . . 117
LIST OF TABLES

I. The binding of Fd11F to Fd. ................................. 52

II. Comparison of the suppressive capability of Fd11F and A10F in two assays of cellular function. ..................... 68

III. Ability of various fractions eluting from FPLC Superose 12 column to inhibit the in vitro Ab response of Fd-immune spleen cells. ................................................................. 82
LIST OF FIGURES

1. Binding of B16G to Fd11F adsorbed to polystyrene wells 46
2. Binding of Fd11F to Fd adsorbed to polystyrene wells and its detection with B16G. 49
3. The reactivity of B16G and the irrelevant mAb, CAMAL-1, with Fd11F 50
4. The binding of soluble Fd to Fd11F in fluid-phase 53
5. Antigenic analysis of Fd and its analogs using determinant-specific mAb 54
6. Inhibition of Fd11F binding by Fd and its analogs 56
7. Detection of anti-Fd Ab in the SN of spleen cell cultures prepared from B10.D2 mice that were immunized with C-molecule and Fd twice 58
8. Relationship between Absorbance at 405 nm vs. concentration of anti-Fd Ab 60
9A. Specificity of anti-Fd Ab from Fd-immune spleen cell cultures for Fd analogs 63
9B. Specificity of serum anti-Fd Ab from Fd-immune B10.D2 mice for Fd analogs 63
10. The relationship of suppressive activity of Fd11F to its Ag-binding activity 64
11. Specificity of the suppressive activity of Fd11F 67
12. Suppressive activity of affinity-enriched Fd11F compared to BW5147 control material using the "revised" protocol 70
13. X-Y plot showing anti-Fd Ab levels of animals before and after treatment with Fd11F and Ag 72
14. X-Y plot showing anti-KLH Ab levels of animals before and after treatment with Fd11F and Ag 73
15. SDS-PAGE of eluates from B16G affinity columns 76
16. Protein profiles (280 nm) of B16G eluates derived from medium (A), BW5147 (B), and Fd11F (C) SN fractionated on a Superose 12 column using FPLC 78
17. Ag-binding activity of FPLC fractions obtained from Fd11F
(*)', BW5147 (a), and medium (O) elution profiles of Fig. 16

18. SDS-PAGE analysis of FPLC fractions (shown in Fig. 17) to 19 inclusive

19. Ag-binding activity of DC10 and Fdl1F material eluted from slices of a 12% preparative SDS-PAGE gel run under non-reducing conditions

20. PAGE of material from selected fractions of Fig. 19 using a 10% SDS gel run under reducing conditions

21. Two-dimensional gel electrophoresis of the 80k Mr proteins of medium and Fdl1F.
ACKNOWLEDGEMENTS

I would like to thank Anthea Tench Stammers, for both performing the experiments that complemented those presented here and her helpful comments and suggestions.

Special thanks should also go to Agnes Chan and Joan Shellard for their discussions and suggestions about some of the technical aspects of the work. I would also like to acknowledge Jan North, Mark Curry, and Mary MacDonald for their useful discussions about the project.

I have been fortunate to receive enormous support from both my families during this time. The knowledge, encouragement and guidance received from my supervisor, Julia Levy will always be remembered. Lastly, I thank my wife, Esther, for her understanding and belief in me.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CS</td>
<td>contact sensitivity</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T cell</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-dinitrophenyl</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>FPLC</td>
<td>high performance gel filtration</td>
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<tr>
<td>Fd</td>
<td>ferredoxin</td>
</tr>
<tr>
<td>GAT</td>
<td>$1\text{-glutamic acid}^{60}\text{-l-alanine}^{30}\text{-l-tyrosine}^{10}$</td>
</tr>
<tr>
<td>HPLC*</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>IEF*</td>
<td>isoelectric focusing</td>
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<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>$M_r$</td>
<td>relative molecular mass</td>
</tr>
<tr>
<td>NP</td>
<td>4-hydroxy-3-nitrophenyl acetyl</td>
</tr>
<tr>
<td>OFd</td>
<td>peroxyformic acid-oxidized ferredoxin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>RaMIG-AP</td>
<td>a conjugate of rabbit anti-mouse immunoglobulin and alkaline phosphatase</td>
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s.c. subcutaneous
SDS-PAGE* sodium dodecyl sulphate polyacrylamide
gel electrophoresis
SN supernatant
TCR T cell receptor
Th T helper cell
Ts T suppressor cell
TsF T cell suppressor factor

Monoclonal antibody

Specificity

B16G invariant region of T cell suppressor factor
CAMAL-1 leukemia-associated antigen
16a carboxyl-terminal determinant of ferredoxin
6-90 amino-terminal determinant of ferredoxin

Abbreviations marked with an asterisk were considered as standard forms and were not defined in the text. The forms of abbreviations were taken from the standard list published by the Journal of Immunology (J. Immunol. 1988. 140 (4).)
INTRODUCTION

Suppression and the Immune Response

The manner in which the immune system regulates itself following stimulation by antigen (Ag) is not well understood. It is known that the response is modulated by both T helper cells (Th) and T suppressor cells (Ts). The study of Th has been aided by the elucidation of the structure of its clonally distributed receptor, the $\alpha/\beta$ T cell receptor (TCR), and the ability to clone these cells directly.

In contrast, the study of suppression has been hampered by the relative difficulty in cloning T cells with suppressive capability. Furthermore the inability to identify $\beta$ chain rearrangements from Ts hybridomas or cell lines has led to the suggestion that suppressor cells may possess an alternate type of TCR (Hedrick et al. 1985).

In spite of this, progress has been made in the characterization of the Ts subset. One intriguing aspect that has received much attention, is the description of Ag-specific T cell suppressor factors (TsF): soluble analogs of the Ts that appear to share many of its properties, such as Ag specificity and the capacity to suppress the immune response. TsF have been described at both the functional and biochemical levels; however, a consensus has not been reached concerning their precise molecular nature and actual relationship to the Ts.
Properties of T cellSuppressor Factors

The existence of soluble T-cell derived material capable of both Ag-binding and suppressive activity is substantially documented. Initially, TsF was prepared as crude thymus or spleen extracts obtained from mice that were primed with carrier Ag without adjuvant. This method of immunization favored the induction of Ts. For example, Takemori and Tada (1975) showed that suppression of the anti-hapten antibody (Ab) response was possible when mice were immunized with the hapten-carrier conjugate (using adjuvant) and given the spleen extract at the same time. This demonstrated that the extract contained suppressive material that was directed to the carrier, in this case, keyhole limpet hemocyanin (KLH) and not the hapten (2,4-dinitrophenyl, DNP) portion of the immunogen. Furthermore, the response was carrier-specific since an extract prepared from KLH-primed mice would not suppress the anti-DNP Ab response of mice made immune to DNP conjugated to a different carrier, bovine gamma globulin (BGG). Only an extract obtained from BGG-primed mice would now effect the suppression of the anti-DNP Ab response. The suppressive activity of KLH-specific TsF was also genetically restricted: ie. genetic identity between the TsF donor and the TsF recipient was required, at a certain gene locus, known as I-J (see below), for suppression to occur. Immunochemical analysis indicated that the TsF could be bound by Ag and by an antiserum raised to the Major Histocompatibility
Complex (MHC) of the TsF donor.

Later work showed that Ag-specific TsF expressed determinants that were encoded by the I-J subregion, a genetic locus that mapped supposedly to within the I region of the murine MHC (Murphy et al. 1976; Tada, Taniguchi, and David 1976). The MHC or H-2 complex, is located on chromosome 17 of the mouse. The gene products from the I subregions, I-A and I-E, play a fundamental role in governing immune responsiveness because Ag is recognized by the Th only in association with these cell-surface proteins, also known as class II antigens. Paradoxically, molecular genetic analysis had demonstrated that I-J encoded gene products could not have actually originated from within the I region (Kronenberg et al. 1983). At present, the origin of the I-J product(s) remains unknown but I-J determinants, as recognized by antisera or monoclonal antibodies (mAb), are still understood as markers for Ts and TsF (Murphy 1987; Dorf and Benacerraf 1985).

The H-2 linked nonresponder status of certain murine strains to specific Ag has frequently been associated with the production of both Ts and TsF. For example, H-2^q animals do not mount an Ab response to the synthetic terpolymer, 1-glutamic acid\(^{60}\)-l-alanine\(^{30}\)-l-tyrosine\(^{10}\) (GAT). This nonresponsiveness was found to be mediated by Ts (Kapp et al. 1974). However, H-2^q mice became responsive to GAT when they were immunized with GAT that was covalently linked to methylated BSA (GAT-MBSA). Kapp et al. (1976) demonstrated that thymus and spleen extracts from GAT-
primed mice suppressed the Ab response of GAT-MBSA-immunized mice if the extract was administered at the same time as Ag. In vitro suppression of anti-GAT Ab production by TsF was shown by the addition of extract to a primary culture of spleen cells that was stimulated with GAT-MBSA. Specificity of suppression was shown by the inability of GAT-TsF to suppress the in vitro response to an irrelevant Ag. Like KLH-TsF, GAT-specific TsF expressed I-J encoded determinants and could be bound by immobilized Ag.

Other studies have shown that T cell-mediated suppression was not achieved by just one type of Ts. In general, suppression was accomplished through several phenotypically different T cell subsets, each producing its own type of TsF or mediator.

Cantor and Gershon (1979) proposed that T cell subsets could be distinguished by both the expression of cell surface glycoproteins (called Ly markers) and their functional properties. They described Ly1+2−, I-J+ inducer cells, that induced resting nonimmune T cells to exert suppressive activity, Ly1−2+ effector cytotoxic/suppressor cells, that could be further classified according to their I-J determinant expression (cytotoxic cells being I-J−, suppressor cells being I-J+) and Ly1,2+ regulatory cells, that were the targets of the inducer cells. Yamauchi et al. (1981a,b) reported that Ly1+2− cells secreted an Ag-specific TsF that was targeted for Ly2+ cells. Ly1−2+ cells, in turn, produced a second factor (effector TsF) that caused suppression. Taniguchi and Tokuhisa (1979) also reported that at least one other type of T cell was required, in
addition to the one producing Ag-specific TsF, to cause suppression of the anti-hapten Ab response. This other cell (effector Ts) was the target of the Ag-specific TsF and required that the cells be primed with Ag. However, once these effector Ts were combined with TsF, in the presence of Ag, they were capable of suppressing the Ab response to other Ag as well.

According to Hausman, Sherr, and Dorf (1986) for the 4-hydroxy-3-nitrophenyl acetyl (NP) hapten system, suppression was achieved through a cascade of at least three phenotypically different T cell subsets that each secreted its own type of TsF. Thus, immunization of mice with NP-coupled spleen cells induced a primary level of Ts known as first order Ts (Ts₁). These cells secreted TsF₁ that bound NP, expressed I-J determinants and also expressed idiotypic determinants associated with the NP-binding structure of the TsF. Just as Ab can possess antigenic determinants located within the Ag-combining site of the Ab molecule (idiotype), T cell receptor structures can have idiotypic determinants. TsF₁ activity was demonstrated by its ability to suppress the contact sensitivity (CS) response to NP in mice that were either syngeneic or allogeneic with the TsF donor. TsF₁ would only suppress if administered during the initial priming of the animal with Ag. TsF₁ also induced second level or second order Ts (Ts₂) in animals. In this way, TsF₁ is the inducer TsF described above. This did not require the presence of exogenous Ag. Ts₂ secreted TsF₂ that differed from TsF₁ in several aspects: TsF₂ was anti-idiotypic and bound to
anti-NP Ab ie. the idiotypic determinants of the Ab. TsF$_2$ expressed I-J encoded determinants and could suppress the CS response of previously primed animals. Furthermore, it was genetically restricted for suppressive activity. Lastly, third order Ts (Ts$_3$), were primed at the same time as Th during Ag exposure, but were only activated by TsF$_2$. TsF$_3$ bound to NP, expressed I-J encoded and idiotypic determinants of the NP-binding moiety. The final target of the TsF$_3$ was the T cell responsible for mediating CS. Once NP-specific TsF$_3$ interacted with NP, the suppression of other CS responses in the immediate vicinity occurred (bystander suppression). It is not known if actual physical contact between the cell types is required for the expression of suppressive activity. However the idiotypic determinants of Ts$_3$ and Ts$_1$ and the anti-idiotypic specificity of Ts$_2$ (TsF$_2$) could be viewed as a possible way of bridging all three suppressor components together. Other suppressor pathways have been proposed and are reviewed by Tada (1984).

A more detailed description of the functional properties and molecular composition of TsF became possible with the development of T cell hybridomas and clones. Factor-secreting T cell hybridomas and clones served as homogeneous and continuous sources for the biochemical study of TsF. It has been estimated that TsF in µg quantities can be recovered from the hybridoma supernatant (SN) instead of in ng quantities from mice (Krupen et al. 1982).

Ts hybridomas were created via the fusion of the BW5147
thymoma with spleen cells enriched for Ts activity and (or) the expression of Ts markers (eg. Taniguchi, Saito, and Tada 1979; Kapp, Araneo, and Clevinger 1980). Ts clones were derived from spleen cells enriched for Ts activity and (or) the expression of Ts markers, and then propagated using interleukin-2, (IL-2) (eg. Fresno et al. 1981; Nakauchi et al. 1984).

TsF, specific for KLH, produced from a Ts hybridoma that expressed I-J encoded determinants and bound to Ag-coated plates, demonstrated the same properties as the suppressive material extracted from spleen cells, ie., Ag-binding, genetically restricted in activity, and I-J\(^+\). Gel filtration showed that TsF had a relative molecular mass (M\(_r\)) of 42k-68k. A comparison of the TsF material prepared from SN (secreted form) or cellular extracts (cytoplasmic form) demonstrated that this particular TsF was composed of two chains: an Ag-binding one and an I-J determinant-bearing one (Taniguchi, Saito, and Tada, 1979; Taniguchi, Takei, and Tada 1980).

An effector TsF, so called for its ability to act directly on Ly1\(^+\) Th to suppress the anti-KLH Ab response, was prepared from a cloned Ts line (Kitamura et al. 1984; Nakauchi et al. 1984). This KLH-specific TsF was phenotypically different from the previously mentioned monoclonal KLH-TsF in that it did not demonstrate MHC restriction for its expression of activity and it did not express I-J coded determinants. Unlike the other effector TsF mentioned (TsF\(_3\), Hausman, Sherr, and Dorf 1986) it was Ag-specific in action. It was also clearly shown that the
simultaneous presence of Ag and TsF were required for suppression to occur.

A GAT-specific TsF from a Ts hybridoma has also been reported (Kapp, Araneo, and Clevinger 1980). This TsF demonstrated the same properties as the material extracted from spleen cells (Kapp et al., 1976); i.e., binds Ag, expresses I-J encoded determinants, and suppresses the primary response to GAT-MBSA-primed cells. In addition, the T cell proliferative response of mice primed with GAT-MBSA was inhibited when TsF was added to cultures containing GAT. The effector KLH-TsF of Kitamura et al. (1984) was also able to suppress the T cell proliferative response of Ag-primed cells. In this case, the target of the TsF was shown directly to be Ly1+ cells since incubation of Ag-primed Ly1+ cells with Ag and TsF resulted in the inhibition of T cell proliferation.

Ts hybridomas have facilitated the biochemical study of TsF. In practically all cases, hybridomas were selected for the ability to constitutively secrete active material into the SN.

Krupen et al. (1982) purified the GAT-specific TsF of Kapp, Araneo, and Clevinger (1980, see above) to homogeneity. Using affinity chromatography, two rounds of reverse-phase HPLC and ion-exchange HPLC, they recovered 1.8 μg of bioactive material from 6 l of crude SN (containing 26 g of protein). When the purified material was analyzed using SDS-PAGE under reducing conditions, one band at 24k M_r was observed. A final purification factor, based on bioactivity and total protein content, was calculated as 5.2 x 10^7.
Using the same GAT-specific Ts hybridoma, Wieder et al. (1982) isolated mRNA from it and achieved cell-free translation of bioactive GAT-TsF. The translated product suppressed both GAT-specific T cell proliferation and the anti-GAT Ab response. It expressed I-J determinants, and when purified to homogeneity using affinity chromatography, reverse-phase HPLC and SDS-PAGE, the TsF resolved as a 19k $M_r$ protein. They explained the discrepancy in $M_r$ with the secreted material (24k $M_r$) as a result of a lack of carbohydrate modification of the factor, since the translating system used (rabbit reticulocyte) did not possess glycosylating activity.

Molecular composition of an effector TsF specific for GAT has also been determined (Turck, Kapp, and Webb 1986). This TsF differed in molecular structure from the previously described GAT-TsF. Effector GAT-TsF was a disulphide-linked heterodimer. When analyzed by two-dimensional non-reducing/reducing SDS-PAGE, one spot, lying off the diagonal of the gel (indicating that it contained a disulphide linkage), was recovered. When the material was applied to a reverse-phase HPLC column, two proteins were resolved. Suppressive activity was restored when they were mixed together and added to culture. Following HPLC separation, one chain was shown to bind Ag while the other chain displayed I-J determinants. Using SDS-PAGE, both chains were resolved at about 30k $M_r$. Like the single chain GAT-TsF, both components of effector TsF were glycosylated. However, in contrast to the single chain factor, bioactivity was lost following neuraminidase
Work with KLH-TsF has progressed in a similar manner to that of GAT-TsF. Taniguchi et al. (1982), using a Ts hybridoma (described by Taniguchi, Takei, and Tada 1979, see above) specific for KLH, obtained several mRNA species that, when translated by the frog oocyte system yielded an Ag-binding chain and a chain expressing I-J determinants. Suppressive activity was recovered when the two chains were combined and tested in culture.

Saito and Taniguchi (1984) biochemically characterized the two-chain KLH-specific TsF. The non-covalently associated chains bound Ag and expressed I-J determinants. The 45k $M_r$ chain bound to both KLH and to a mAb specific for a constant region of TsF. The 27k $M_r$ chain bound to both polyclonal and monoclonal anti-I-JB reagents. Suppression of the anti-hapten Ab response was demonstrated by TsF recovered from immunoadsorbents made with the monoclonal reagents.

Although extensive work has been done with the GAT and KLH systems, other TsF with different specificities have been purified to homogeneity. Webb et al. (1989) described a TsF specific for 1-tyr-p-azophenyltri-methyl-ammonium (TMA). It was described as an inducer TsF that bound Ag, expressed both I-J determinants and the cross-reactive idiotype associated with anti-TMA Ab. Extracted material from the Ts hybridoma was subjected to (NH$_4$)$_2$SO$_4$ precipitation, IEF, reverse-phase HPLC, and high performance gel filtration to yield a major bioactive
peak at 62k $M_r$. Using SDS-PAGE, a major peak was observed at 26k $M_r$. They noted that the biochemical characteristics of TMA-TsF were very similar to that of single-chain GAT-TsF. In fact, they demonstrated that a mAb raised to GAT-TsF bound to TMA-TsF. The authors suggested that both factors belonged to the same family of suppressor inducer proteins.

A TsF isolated from a Ts clone (as opposed to a hybridoma) has been reported. Fresno et al. (1981) recovered a 70k $M_r$, single-chain, Ag-binding factor from a Ly23$^+$ T cell clone. It did not express I-J determinants. It was purified using gel filtration, ion-exchange chromatography and IEF. The target of this TsF was the Th and required the presence of Ag for the expression of suppressive activity.

Summary

A variety of properties have been attributed to TsF. Initially, such features as the specific suppression of the Ab response, the capability to bind Ag, the expression of I-J encoded determinants and the requirement for genetically restricted cellular interactions were discovered using cellular extracts. These findings were verified and extended using Ts hybridomas and the TsF produced by them. Indeed, the linear cascade model for the suppression of the NP response (Hausman, Sherr, and Dorf 1986, see above) was based on data obtained using hybridomas and their respective factors made from the three
orders of Ts: Ts₁, Ts₂ and Ts₃. Other work suggested that suppression was achieved through interacting cell pathways or circuits with each phenotypically distinct Ts subset elaborating its own type of TsF (eg. inducer TsF, effector TsF). The target of the Ag-specific TsF was either the next Ts in the pathway or the terminal cell of the path, the effector T cell, which mediated the function that was under suppressive control (eg. Th). Since TsF and Ts can express idiotypic determinants, one possible way of cell to cell recognition was through the idiotype-anti-idiotype network. Genetic restriction could also help to insure that the appropriate cells and factors would interact. Although suppression of the effector function was Ag-dependent and specific, other T cell responses in the immediate vicinity would be suppressed (bystander suppression). This explains how a monoclonal TsF could suppress a polyclonal T cell response. Factor-secreting Ts hybridomas have also made it possible to study TsF at the molecular level. Several TsF have been purified using different separation techniques including, affinity and ion-exchange chromatography, IEF and reverse-phase HPLC, in various combinations, to yield homogeneous and bioactive material. TsF has been isolated as a one chain or two chain molecule. The one chain TsF was bioactive, bound Ag and expressed I-J encoded determinants. The two chain structure consisted of an Ag-binding chain and a non-Ag-binding chain that displayed I-J determinants. Both of the chains were required for bioactivity and have been reported as either non-covalently
associated or disulphide-linked. Messenger RNA has been prepared from at least two different Ts hybridomas and used to achieve translation of bioactive TsF. Both the one chain and two chain types of TsF have been synthesized using in vitro translation techniques.

A precise description of the TsF molecule has been difficult because of the variety in both its reported molecular and functional properties. Whether the variety of results is a reflection of the different Ag systems under study, the labile nature of the TsF molecule, the complexity of the suppressor mechanisms at work or a combination of all three, is unknown. Despite the effort being devoted to the study of TsF, a consensus on its structure and function will only be reached only by stringent testing eg. molecular cloning of a gene(s) encoding an Ag-specific TsF.

The Immune Response to Ferredoxin as a Model for the Study of Mechanisms of Immunoregulation

The immune response to ferredoxin (Fd) has been studied extensively in this laboratory. Collectively, the findings have served as a defined model for the immune response to a natural protein Ag.
The choice of Fd as a model antigen

Fd is a small (5500 M<sub>r</sub>) molecule produced by <i>Clostridium pasteurianum</i> which functions as an electron-acceptor protein in the electron transport pathway of that organism. It has been well-characterized biochemically and purified to homogeneity (Rabinowitz 1972). The amino-acid sequence of Fd was confirmed using gene cloning techniques (Graves, Mullenbach, and Rabinowitz 1985) and its three-dimensional structure has been determined using X-ray crystallography (Jensen 1974). This 55 amino-acid protein is distinguished by the presence of eight cysteine residues, the sulfur atoms of which, form coordination bonds with two iron-sulfur clusters, that make up the central core of the protein. It has been hypothesized that Fd was created through a gene duplication event as the protein has a two-fold axis of symmetry and 13 identical pairs of amino-acid residues that are present in both halves of the molecule.

In addition to having knowledge of its biochemistry, there was another reason for choosing Fd as a model Ag for the study of immunoregulatory mechanisms. Stated simply, Fd is a natural protein Ag of defined structure. This type of molecule has two distinct advantages over some of the other experimental Ag. Firstly, natural proteins are what is encountered by the immune system under physiological conditions and therefore any mechanisms elucidated by its study would presumably have broad based implications. Such conclusions cannot necessarily be drawn
from synthetic haptens or Ag. Secondly, natural proteins are of defined primary structure, as opposed to synthetic Ag like GAT, or derivatized antigens like DNP-KLH. Knowledge of the precise antigenic structure is vital for the study of immunodominance: the phenomenon in which, of the potentially large number of epitopes expressed by the Ag, only a limited number are selected to interact with lymphocyte clones. Having a defined structure allows the identification of these immunodominant epitopes. As will be described below, Fd has been useful as a prototype for the study of the immune response to T cell-dependent natural protein Ag. Some of the work with Fd has involved the use of derivatized or synthetic forms of the Ag. However the data have always been related to or interpreted in terms of the response to the native Ag.

Epitope mapping and H-2 linked responsiveness to Fd

Initial studies were aimed at mapping the major antigenic determinants of Fd using both rabbit antiserum and Ag-primed T cells from guinea pigs. It was demonstrated that Fd contained a limited number of determinants since peroxyformic acid modification of the cysteine residues of the native molecule did not alter the binding of antiserum raised to the unmodified protein (Nitz et al. 1969). Further work, by Kelly and Levy (1971), using rabbit antiserum raised to peroxyformic acid-treated Fd (OFd) and synthetic peptides representing various
sequences in the Fd molecule showed that essentially all of the Ab activity was directed to either the amino-terminal heptapeptide (NH$_2$-ala-tyr-lys-ile-val-asp-ser-COOH, known as N-determinant) or the carboxyl-terminal pentapeptide (NH$_2$-ala-pro-val-gln-glu-COOH, known as C-determinant). The ability of both the amino-terminal and carboxyl-terminal peptides to act as T cell determinants was shown using synthetic peptides consisting of the immunodominant sequences linked together via a repeating glycine bridge. The synthetic peptides proved to be immunogenic in guinea pigs since the T cells obtained from these primed animals would proliferate in response to the respective peptides as well as to the intact Ag (Levy et al. 1972; Kelly, Levy, and Hull 1973). Evidence that the N-determinant and C-determinant-primed cells synergized for the generation of a Th cell response was provided when it was demonstrated that only a mixture of the two separately primed cell populations would recognize the hapten-carrier complex, DNP-OFd in an anti-hapten (DNP) Ab response (Feldmann, Kilburn, and Levy 1975).

Sikora and Levy (1980) carried out a genetic analysis using inbred strains of mice and determined that the Ab response to Fd was H-2 linked. Using congenic strains of mice, the response was mapped to the I-A subregion. H-2$^k$ animals were identified as high responders, H-2$^{b,s}$ mice as intermediate responders and H-2$^d$ animals were designated as nonresponders.

An important study by Sikora, Weaver, and Levy (1982) confirmed and extended the previous work that described the
determinant mapping of Fd. They showed that Fd consisted of two antigenic determinants, through the use of structural analogs which were generated by enzymatic digestion. Trypsin cleavage of Fd destroys the N-determinant by removing the terminal tripeptide, leaving the remainder of the molecule, including the C-determinant, intact. Carboxypeptidase A partially digests the C-determinant by removing the terminal 3 amino acids, leaving the rest of the molecule, including the N-determinant, intact. A doubly digested molecule did not react with anti-Fd antiserum prepared from high responder mice, confirming the earlier observation that Ab activity was directed exclusively to the two determinants. Monoclonal antibodies with specificity for either the N-determinant or C-determinant were produced and shown to bind to the respective enzyme-digested molecule bearing the appropriate determinant. It was also observed that the sum of the N-determinant and C-determinant reactivities of an antiserum was equal to its total anti-Fd reactivity. This finding was used as basis of comparison between strains of mice that were responsive to Fd. The high responding strains (I-A\textsuperscript{k}) produced Ab, of which, 60 to 80% was directed to the C-determinant. Of the intermediate responders (expressing the b and s alleles at the I-A subregion), only 25 to 50% of the total anti-Fd Ab was specific for the C-determinant.

It was found that injection of nonresponder mice (H-2\textsuperscript{d}) with trypsin-digested Fd (also referred to as C-molecule) made them responsive to subsequent challenges of Fd (Sikora and Levy 1984).
Furthermore, if the mice were primed with carboxypeptidase A-treated Fd (also referred to as N-molecule) and then challenged with C-molecule, this resulted in an accelerated anti-Fd Ab response as compared to control animals that received C-molecule immunization only. It was postulated that a second overlapping determinant resided within the N-determinant heptapeptide region that was "masked" by the ala-tyr-lys tripeptide, but was exposed in the case of C-molecule. Interestingly, for the C-molecule-primed mice, less than 10% of the anti-Fd Ab was specific for the C-determinant (see Fig. 9B).

Summary

Fd was chosen as a model Ag because it is a small, natural protein with defined structure. Initial studies identified the amino-terminal heptapeptide and the carboxyl-terminal pentapeptide as the only antigenic determinants or epitopes of the molecule. These determinants were reactive with both Ab and T cells. This suggested that either epitope had the potential for being assigned a hapten or carrier function. Monoclonal antibodies were raised to either determinant and through the use of analogs helped to verify the original epitope mapping of Fd. The immune response to Fd was observed to be H-2 linked and determinant selectivity of the anti-Fd Ab response was also related to the haplotype (genotype) of the responding mouse. H-2K animals were identified as high responders and 60 to 80% of
the Ab response was directed to the C-determinant. H-2^{b,s} animals were designated as intermediate responders and 25 to 50% of the Ab was specific for the C-determinant. Lastly, tolerance to Fd, expressed by the nonresponder H-2^d mice, could be broken by priming the animals with C-molecule, the trypsin-digested analog of Fd. Less than 10% of the anti-Fd Ab of these animals was directed to the C-determinant.

Idiotype-anti-idiotype interactions

The study of the immune response to Fd has also been pursued by defining and examining idiotypic-anti-idiotypic interactions associated with the anti-Fd response. The concept of the immune system as an autoregulatory network with components that recognize each other via sets of antigenic determinants expressed by the V regions of Ab or TCR (idiotype), is now generally accepted.

Network interactions that influenced the immune response to Fd were first revealed through the use of an anti-idiotypic reagent (rabbit antiserum) specific for the idiotype expressed by the anti-Fd mAb, Fd-1. This mAb is specific for the N-determinant of Fd. The idiotype identified by the anti-Fd-1 reagent represented a major idiotype expressed in anti-Fd Ab obtained from both H-2^k high responders and H-2^s intermediate responders (Weaver, Sikora, and Levy 1982).

Weaver et al. (1983) described T cells, which expressed the
Fd-1 idiotype, that helped to regulate the anti-Fd Ab response of B10.BR (H-2^k) animals. This was done by treating B10.BR T cells with anti-Fd-1 plus complement and then adoptively transferring them to sublethally irradiated B10.BR recipients. The recipients were immunized with Fd and monitored for Ab production. There was a significant increase in anti-C-determinant-specific Ab in the animals that received T cells treated with the anti-Fd-1 reagent as compared to control animals. This suggested that a population of Fd-1 idiotype-bearing T cells was being influenced by the anti-idiotypic reagent. Interestingly, the level of anti-N-determinant Ab or the percentage of expression of the Fd-1 idiotype in the antiserum did not change with the anti-idiotype treatment. When Fd-1, the mAb displaying the Fd-1 idiotype, was administered to nonimmune B10.BR mice seven days prior to Fd immunization, there was a significant increase in anti-Fd Ab production in those animals that had received the mAb injection. This suggested to the authors that an anti-idiotypic population of T cells also existed and that the interaction between the idiotype-bearing and the anti-idiotype bearing T cells was important in the regulation of the anti-Fd Ab response in B10.BR animals.

Singhai et al. (1984) characterized another anti-Fd mAb, Fd-2, that was specific for the C-determinant of Fd and prepared an anti-idiotypic reagent to it. When this anti-Fd-2 idiotype reagent was administered to B10.BR mice, that were subsequently immunized with Fd, the anti-Fd Ab response was enhanced in
treated animals as compared to control groups. The level of anti-C-determinant Ab had increased, as was observed by Weaver et al. (1983). Unlike the Fd-1 idiotype, the Fd-2 idiotype was not detected in B10.BR immune sera. More interestingly, the administration of anti-Fd-2 to nonresponder B10.D2 mice, that were then immunized to Fd, resulted in the production of anti-Fd Ab in these animals. This abrogation of nonresponsiveness was investigated further by Singhai, Hoffmann, and Levy (1985). They treated B10.D2 T cells with either Fd-2 and complement or anti-Fd-2 and complement and adoptively transferred the remaining cells into syngeneic irradiated B10.D2 recipients. The recipients were immunized with Fd and monitored for the production of anti-Fd Ab. Both groups of animals responded to the Fd challenge. Reconstitution of nonresponsiveness was demonstrated by adoptively transferring a mixture of T cells that was composed of the Fd-2-depleted suspension and the anti-Fd-2-depleted suspension into recipients, that were then challenged with Fd. The anti-Fd Ab levels of these animals were negligible as compared to the other immunized recipients who received either the Fd-2-depleted or the anti-Fd-2-depleted T cells. This suggested that both populations of T cells expressing the Fd-2 idiotype and the anti-Fd-2 idiotype were required for the maintenance of the nonresponder status in H-2d animals.
Summary

Idiotype was defined as the set of antigenic determinants expressed by the V regions of Ab or TCR. Idiotypic-anti-idiotypic interactions were investigated using anti-Fd mAb as the idiotype-expressing reagents. Affinity-purified anti-idiotypic antisera against these idiotypes were raised in rabbits. The Fd-1 idiotype was expressed in the majority of sera tested from high and intermediate responder animals. This idiotype was also expressed by a population of B10.BR T cells that appeared to regulate the anti-Fd Ab response. The observation that an enhancement in anti-Fd Ab in B10.BR animals was caused by the administration of Fd-1 idiotype itself suggested that anti-Fd-1-idiotype-bearing cells existed as well. It was speculated that the idiotype-bearing and anti-idiotype-bearing cell populations worked in concert to help regulate the Ab response in B10.BR animals. Results from the investigation of a second immune network interaction (designated Fd-2 idiotype) in B10.D2 mice showed that two populations of idiotype-bearing and anti-idiotype bearing T cells governed responsiveness to Fd. When either population was depleted, responsiveness ensued following immunization. When the two populations were combined, and returned to the animal almost complete restoration of nonresponsiveness resulted.
B16G and the Development of the Fd11 hybridoma

Another area of interest in this laboratory has been the development and use of B16G, a mAb having apparent specificity for an invariant epitope on TsF. B16G has been used for both the screening of Ts hybridomas and the isolation of suppressive material using immunoaffinity columns. In order to extend our studies of the immune response to Fd, a T cell hybridoma, Fd11, was raised against that Ag and screened using B16G.

B16G itself was developed as a result of studies on the P815 tumor system. The murine mastocytoma P815 is a well characterized tumor model which is lethal to DBA/2 mice when injected at relatively low doses. It was shown that soon after tumor inoculation (three to five days) a P815-specific CTL response was evident. However this phase was quickly overwhelmed by the emergence of Ts. The appearance of Ts correlated with an acceleration in tumor growth (Takei, Levy, and Kilburn 1976). Other workers have also described the involvement of Ts in the immune response to this and other tumors (Granstein et al. 1984; Fisher and Kripke 1982; Mills and North 1985).

Further investigation led to the identification of a soluble factor, extractable from the thymocytes of P815-bearing mice, that was capable of suppressing the syngeneic in vitro cytolytic T cell (CTL) response to P815 (Takei, Levy, and Kilburn 1978). The factor appeared to have a degree of tumor specificity. The suppressive activity could be absorbed out of solution by passage
over a P815-Sepharose column but not by a control column. Attempts at purification of this material were not successful.

In order to study tumor-specific factors more directly, Maier, Stammers, and Levy (1983) developed and characterized the TsF-specific mAb, B16G. Affinity-enriched thymocyte-derived TsF was used to immunize mice from which B16G was derived. Once it was isolated and cloned, B16G was tested both in vivo and in vitro. Pretreatment of DBA/2 mice with B16G not only inhibited the growth of P815 tumor but the syngeneic tumor, M-1 as well. That B16G was nonspecifically immunoenhancing was also substantiated by noting that DBA/2 spleen cells from B16G-treated animals responded better to irradiated histoincompatible cells by proliferation than did untreated controls. This enhancing effect on T cell proliferation suggested that B16G was responsible for the inactivation of a regulatory (suppressor?) T cell. Panning experiments, where T cells were incubated on or "panned over" B16G-coated plates, demonstrated that B16G was responsible for the removal of a regulatory T cell from the spleen population.

With the availability of a "TsF"-specific mAb for selection purposes, a T cell hybridoma, A10, was created from the murine thymoma line, BW5147 and T cells stimulated in vivo with P815 membrane extracts (Steele, Stammers, and Levy 1985). A10 factor (A10F), was recovered from SN using either B16G- or P815-Sepharose columns. When A10F was administered intravenously (i.v.) to DBA/2 mice, it caused the enhancement of P815 tumor growth, but not that of L1210 or M-1 tumor. In addition, A10F
did not enhance or suppress the T cell proliferative allogeneic response of DBA/2 spleen cells. The inability of A10F to affect the growth of two unrelated tumors or the T cell response of DBA/2 cells to histoincompatible cells indicated that it might be tumor-specific.

Molecular characterization revealed that affinity-enriched A10F resolved as 140k, 80k and 45k M\(_r\) moieties when analyzed by SDS-PAGE under reducing conditions (Steele, Stammers, and Levy 1985; Chan 1988; Chan et al. 1988). The 80k M\(_r\) species had the propensity for "aggregation" to the larger M\(_r\) species or degradation to a smaller size of approximately 30k M\(_r\). SDS-PAGE under non-reducing conditions of A10F showed that suppressive activity was associated with the 140k and 80k M\(_r\) forms while the 45k and 32k M\(_r\) forms showed marginal bioactivity. Biosynthetic labeling of A10 cells with \(^{35}\)S-methionine and cysteine revealed that the 80k M\(_r\) form was actually secreted into the SN and formally ruled out the possibility that the 80k M\(_r\) A10F moiety was a medium or FCS artifact. Amino-acid sequencing of the amino-terminal of the 140k and 80k M\(_r\) forms indicated that they shared the same sequence while the 30k M\(_r\) polypeptide had a different amino-terminal sequence. Because of the possibility that sequence data could have been derived from co-migrating impurities, assignment of these sequences to the TsF molecules could not be done.

Much information has been gathered about the immune response to Fd. Of particular interest is the existence of an idiotype-
anti-idiotype interaction, at the T cell level, which functions as part of the suppressor network to control the immune response to Fd in nonresponder B10.D2 H-2^d mice.

With the knowledge that Fd-specific Ts existed, a T cell hybridoma was made using BW5147 and T cells obtained from Fd-primed B10.D2 animals (Steele, Chu, Chan, North, and Levy et al. 1987). Because the nominal Ag of the Fd11 hybridoma was known, unlike that of the A10 hybridoma, the hybridoma cells were screened for the ability to secrete molecules capable of binding to Fd and B16G. The secreted material, known as Fd11F, could be recovered by affinity chromatography from B16G-Sepharose or Fd-Sepharose columns. Fd11F bound to Fd-coated ELISA plates while control material from an irrelevant hybridoma did not. When analyzed by SDS-PAGE, under reducing conditions, Fd11F-enriched material appeared as two major unique bands, one migrating at about 80k M_r and another at about 30k M_r as well as a number of other minor bands. This was the case whether the material was recovered from either Ag or B16G columns. When Fd11F and Fd were administered to Fd-immune DBA/2 mice concomitantly, the Ab response was suppressed as compared to the control groups receiving PBS or A10F. Evidence that Fd11 can actually recognize Fd, presumably by a cell-associated TCR, was provided by calcium flux studies. The intracellular flux of calcium ions is one of the events associated with a cell surface receptor-ligand reaction. Fd11 cells were loaded with the calcium-sensitive dye, Quin-2. Seconds after the exposure of Fd11 cells to Fd, Quin-2
fluorescence was detected, indicating an increase in the level of intracellular calcium and the presumed binding of Fd by Fd11F expressed on the surface of the cell.

Summary

The study of the immune response to a syngeneic tumor provided the impetus for the development of the mAb, B16G. In the response to the P815 tumor, Ts play the predominant role and allow the growth of the tumor. Soluble extracts with suppressive activity specific for P815 were initially prepared from thymocytes of P815-bearing mice and then later from the A10 hybridoma. Interestingly, B16G was not Ag-specific itself but allowed the selection of Ts hybridomas and the recovery of apparently Ag-specific TsF from these cells. When B16G-reactive material was analyzed using SDS-PAGE, under reducing conditions, proteins of similar $M_r$ were recovered from either the A10 or Fd11 hybridomas. As a corollary of the dual binding of Fd11F to Fd and B16G, the SDS-PAGE appearance of Fd11F was identical whether it was eluted from the Fd- or B16G-Sepharose column. The major advantage that the Fd11 hybridoma offered over the A10 hybridoma was the availability of the nominal Ag of Fd11. Indeed, the study of the Ag-binding properties of Fd11F is a major subject of this thesis.
Purpose of the Investigation

That FdllF was suppressive in vivo and able to bind Ag suggested it might be classified along with the previously mentioned group of proteins known as TsF. This prompted us to study FdllF further with the hope of providing additional insight into the nature of T cell-derived, Ag-binding molecules. Furthermore, an investigation into the properties of FdllF was likely to contribute to the understanding of the anti-Fd response. The present work comprises a detailed examination of FdllF at the immunochemical, biological, and biochemical levels with emphasis on describing the Ag-binding properties of FdllF.
MATERIALS AND METHODS

Buffers and Solutions

The compositions of the buffers and solutions used are listed in the Appendix, THE COMPOSITIONS OF BUFFERS AND SOLUTIONS.

Experimental Animals

B10.BR, DBA/2, B10.D2 and (Balb/c x DBA/2)F₁ mice were purchased from the Animal Care Unit of the University of British Columbia. They were used between 6 and 12 weeks of age.

Cell Lines

The development of the Fd11 hybridoma and a preliminary description of the immunological properties of its secreted product, Fd11F have been described previously (Steele, Chu, Stammers, North and Levy 1987). The Fd11 hybridoma was originally made in 1985 and has remained stable with respect to its production of Fd11F for at least four years. As assessed by fluorescence-activated cell sorter (FACS) analysis, the Fd11 hybridoma was found to be Thy 1⁺ and F23.1⁻. The hybridoma expresses both H-2ᵈ and H-2ᵏ Ag. The DC10 hybridoma was derived by fusion of the BW5147 cell line with T cells obtained from
DBA/2 mice primed to a leukemia-associated Ag and was used as a control in these studies. The hypoxanthine guanine phosphoribosyl transferase negative BW5147 cell line was obtained from the American Type Culture Collection (Rockville, MD). Two cell lines producing the anti-Fd mAb, 16a and 6-90, respectively, were created in this laboratory and have been previously described (Weaver, Sikora, and Levy 1982; Sikora, Weaver, and Levy 1982). The cell line producing the TsF-specific mAb, B16G was also produced in this laboratory (Maier, Stammers, and Levy 1985). The BW5147, DC10 and Fd11 cell lines were tested by the tissue culture service of the American Type Culture Collection and shown to be mycoplasma-free. All cells were maintained in Dulbecco’s Modified Eagle’s medium (Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS, CPSR-3, Sigma, St. Louis, MO), at 37°C, in a 10% CO₂ incubator.

Conventional and Monoclonal Antibodies

The mAb, 16a, 6-90 and B16G were raised as ascites by injecting approximately 5 X 10^6 cells intraperitoneally into (Balb/c X DBA/2)F₁ animals. Three to five days prior to receiving the injection of cells, the mice were pretreated with an intraperitoneal injection of 0.5 ml of 2,6,10,14-Tetramethylpentadecane (Pristane, Aldrich, Milwaukee, WI). Immediately before inoculation with cells, the mice received 250 rads of gamma radiation (Gamma-cell, Atomic Energy of Canada,
Ottawa, Ont.). Tapping of the ascitic fluid was initiated about 10 days after the introduction of cells. Immediately after the collection of fluid, the ascitic cells and debris were pelleted at 400 g for 10 min. The cells were either resuspended in PBS and used to inoculate additional mice, or added to dimethyl sulfoxide and FCS and frozen in liquid nitrogen. The supernatant (SN) was pooled and stored at 4°C. When approximately 25 ml of SN was collected, an equal volume of PBS was added to it. To precipitate the gamma globulin fraction, saturated (NH$_4$)$_2$SO$_4$ was added drop-wise, with constant stirring, to the mAb solution to achieve 50% saturation at room temperature. The solution was stirred at 4°C overnight to complete the precipitation of mAb. The precipitate was spun at 9000 g for 30 min at 4°C. Following centrifugation, the pellet was gently resuspended in PBS to a volume equal to the original supernatant and dialyzed against PBS (2 x 21 changes), at 4°C using 14,000 M$_r$ cut-off dialysis tubing (Spectrum, Los Angeles, CA). Following dialysis, the mAb solution was divided into 50 µl, 1 and 5 ml aliquots and frozen at -70°C. Monoclonal Ab preparations were thawed immediately before use.

Affinity-purified CAMAL-1, a mAb specific for a leukemia-associated Ag, was used as a control preparation, and was supplied by Joan Shellard.

Affinity-purified B10.BR anti-Fd Ab was used as a standard for the measurement of Ab. Antiserum was prepared and passed over a Fd-Sepharose column. After washing the column, Ab was
eluted using 0.1 M HCl, neutralized, dialyzed against PBS and then made into aliquots which were frozen at -20°C. The protein concentration of the Ab solution was measured according to a method modified from Lowry et al. (1951) (see below for details).

**Antigens**

Fd isolated from *C. pasteurianum* (type V, F7629, Sigma), KLH (374805, Calbiochem, La Jolla, CA), bovine serum albumin (BSA, Fraction V, Sigma), lysozyme (Lysf 8D8, Worthington, Freehold, NJ) and cytochrome C (Type III, C-2506, Sigma), were reconstituted with distilled water to a concentration of 1 mg/ml, aliquoted, and stored at -20°C.

0Fd, N-, and C-molecule were prepared from native Fd that had been previously denatured with trichloroacetic acid (TCA-Fd). TCA-Fd was produced by adding an equal volume of 20% TCA to a Fd solution at 1 mg/ml. Precipitation was allowed to continue for 1 hr on ice. Insoluble protein was pelleted at 11,600 g for 10 min. The pellet was washed once with ice-cold 100% ethanol. After centrifugation, the pellet was dried under vacuum.

C-molecule was prepared by dissolving 750 µg of TCA-Fd in 720 µl of 46 mM Tris-HCl buffer, pH 8.1, supplemented with 11.5 mM CaCl₂. Trypsin (TRTPCK, Worthington), at a 1:40 enzyme to protein ratio (w/w; 18.75 µg in 30 µl of 1 mM HCl) was added and the digestion was continued overnight at room temperature with gentle stirring in a sealed vial. N-molecule was prepared by
resuspending 750 µg of TCA-Fd in 720 µl of 25 mM Tris-HCl buffer, pH 7.5, supplemented with 0.5 M NaCl. Carboxypeptidase A (COAPMS, Worthington) was added at a 1:25 enzyme to protein ratio (w/w; 30 µg in 40 µl of 10% LiCl) and digestion was continued overnight at room temperature with gentle stirring in a sealed vial. Following overnight incubation, both digests were aliquoted and stored at -20°C. OFd was prepared as described by Tanaka et al. (1964) by resuspending 750 µg of TCA-Fd in 33 µl of formic acid and 67 µl of performic acid. Performic acid was prepared by mixing nine parts of formic acid with one part of 30% H₂O₂ for 2 hr at room temperature. The Fd solution was reacted for 2.5 hr at -10°C, maintained at this temperature with a 5 M NaCl-dry ice bath. The reaction was stopped by lyophilizing the protein.

**Enzyme-linked Immunosorbent Assay**

The amount of anti-Fd Ab present in the spleen cell culture SN was measured using ELISA. Polystyrene ELISA wells (Immulon I, Dynatech, Alexandria, VA) were coated with 100 µl of Fd solution prepared at 1 µg/ml in bicarbonate buffer, overnight, at 37°C, under humid conditions. Between each incubation step, the plates were washed three times using PBS-Tween. After washing, 100 µl of SN were added to the wells and allowed to react for 2 hr at 37°C under humid conditions. The plates were washed again and 100 µl of rabbit anti-mouse immunoglobulin-alkaline phosphatase
(RaMIg-AP, Jackson, West Grove, PA), used at 2 µg/ml, diluted in PBS-Tween, were added and allowed to react for 1 hr under the same conditions as above. After washing, 100 µl of p-nitrophenyl phosphate substrate (one tablet/5 ml buffer, 104-105, Sigma) in diethanolamine buffer were added.

Fd11F was also measured using solid-phase ELISA. Fd-coated plates were prepared as above. Fd11F or control material at various concentrations diluted in PBS, or fractionated material containing Fd11F were added in 100 µl volumes to Fd-coated wells for 2 hr at 37°C under humid conditions. After washing, 100 µl of the TsF-specific mAb, B16G, diluted in PBS-Tween, was added at 18 µg/ml, and incubated for 1 hr at the conditions described above. RaMIg-AP and substrate were used as mentioned previously.

The inhibition of Fd11F binding to Fd-coated wells by fluid-phase Fd was measured using ELISA. Various concentrations of Fd, its analogs, or the control, cytochrome C diluted in PBS, were added to an equal volume of Fd11F solution, used at a concentration of 16 µg/ml. The mixture was gently shaken and incubated at 4°C for 2 hr. The amount of free Fd11F remaining in solution was then determined using the ELISA described above.

For all three immunoassays, the substrate was developed for 1 to 3 hr at 37°C under humid conditions. The plates were read using an automated ELISA reader (Multiskan, Flow, Mississauga, Ont.) set at 405 nm. Each ELISA determination was performed in triplicate and the average value was used for data calculations.
Immunization of Animals

B10.D2 animals were immunized to Fd using C-molecule followed by a boost with Fd. The mice were primed with 50 µg of C-molecule in PBS prepared as a 1:1 (v/v) emulsion with complete Freund's adjuvant (H37 Ra, Difco, Detroit, MI) in a total volume of 0.1 ml. The immunization was administered subcutaneously (s.c.) in the flank on the ventral side. After 21 days, the secondary immunization was given in an identical manner with the exception that 50 µg of Fd were prepared using incomplete Freund's adjuvant (Difco) and the site of injection was in the opposite flank to that of the first injection. Hyperimmune B10.BR animals were prepared in a similar manner with the exception that both the primary and secondary immunizations were with Fd.

Animals were immunized with 50 µg of lysozyme followed by a boost 21 days later. Antigen emulsion and route of administration were as for Fd.

Co-immunization of DBA/2 animals with Fd and KLH was accomplished by giving the Ag separately to opposite sides of the mice. KLH was administered at 50 µg/mouse.

Enrichment of Fd11F using Immunoaffinity Chromatography

Fd11F-enriched material was recovered from the SN using immunoaffinity chromatography. Cultures of Fd11 hybridoma cells
were grown to a density of about $5 \times 10^5$ to $1 \times 10^6$/ml. Typically, 2 l of cells were grown for each lot of Fd11F. Supernatant was recovered by centrifugation using a Sorvall GS-3 rotor (Dupont, Newtown CT) spun at 4,300 g for 20 min. Supernatant was pumped onto a column (2.5 x 23 cm, Econo, Bio-Rad, Richmond, CA) at a flow rate of 60 ml/hr, containing 5 ml of B16G-Sepharose CL-4B beads. B16G was coupled to Sepharose CL-4B (Pharmacia, Montreal, P.Q.) using CNBr (F964-5, Baker, Phillipsburg, NJ) as described by Cuatrecasas (1970). However, just before SN was pumped onto the column, the beads were preconditioned with 5 ml of 0.1 M HCl followed by washing with 200 ml of PBS. Once the SN had been loaded, the beads were washed with PBS at a rate of 100 ml/hr until the absorbance (280 nm) of the effluent was less the 0.005. Protein was eluted using 10 ml of 0.01 M HCl. The entire 10 ml of eluate was collected and then neutralized by adding 1.5 M Tris-HCl, pH 7.5, 100 µl at a time. This was continued until the pH of the solution reached 7.5. Following neutralization, the solution was immediately dialyzed against PBS and then concentrated using microconcentrators (30k $M_r$ cut-off, Centricon-30, Amicon, Danvers, MA). After concentration, aliquots of Fd11F-enriched material were prepared using polypropylene tubes and then frozen at -70°C. If the sample was to be used the next day for preparative, non-reducing SDS-PAGE or high performance gel filtration analysis, then it was concentrated to 500 µl or 200 µl, respectively, and stored at 4°C until use. Samples used for
biological testing in vitro were dialyzed against RPMI 1640 instead of PBS. Control material was prepared concurrently with Fd11F using a duplicate set of equipment. All procedures, including affinity chromatography, were conducted at 4°C.

The protein concentration of the B16G-eluted material was determined by a modification of the method of Lowry et al. (1951). The protein sample was dissolved in 200 µl of distilled water. This solution was then mixed with 200 µl of 1 N NaOH. Two separate stock solutions of 2% Na₂CO₃ in distilled water and 0.5% CuSO₄·5H₂O in 1% sodium citrate were prepared and portions of each were mixed in a ratio of 49 parts (Na₂CO₃ solution) to 1 part (CuSO₄ solution), v/v. Two millitres of this solution were added to the protein mixture, shaken immediately and after exactly 5 min, 200 µl of 1 N Phenol reagent (Fisher, Fairlawn, NJ) were added. Again, the tube was shaken immediately. The reaction was then developed for 30 min. At the end of this time, the colour of the solution was read at 750 nm. A series of BSA solutions, containing from 2.5 to 50 µg of protein, was used for the standard curve.

In vitro Measurement of Fd11F Suppressive Activity

Fd-immune B10-D2 mice were killed 13 to 15 days following the booster immunization with Fd. Spleens were removed aseptically and made into single cell suspensions by passage through a wire screen. The cells were washed three times using RPMI 1640 (10-
601-24, Flow, Mclean, VA) supplemented with 10% FCS and penicillin and streptomycin (100 units/ml and 100 ug/ml, respectively, Gibco) and spun at 400 g.

a) "Original" protocol: After washing, the cells were pulsed with Fd which had been previously sterilized using a 0.22 µm filter (4192, Gelman, Ann Arbor, MI). Prior to pulsing, about 1 X 10^7 cells were placed into a separate tube and did not receive Fd. Pulsing conditions were 250 µg Fd/1.5 ml medium/spleen. The pulsing medium was supplemented with FCS to a final concentration of 20%. The pulsed and unpulsed cells were cultured at 37°C in a 10% CO_2 atmosphere for 2 hr using polypropylene tubes. The cells were resuspended gently twice during the 2 hr period. After washing, the cells were adjusted to a concentration of 2.5 X 10^6/ml, supplemented with 2-mercaptoethanol (M 6250, Sigma) to a final concentration of 50 µM, and plated out as 2 ml cultures. Fd11F or control material was added at this time. The cells were cultured for seven days at 37°C in a 10% CO_2 atmosphere. Culture SN were carefully removed on day 7 and undiluted samples were measured for Ab content using ELISA.

b) "Revised" protocol: Cells were pulsed initially with Fd11F or control material, instead of Fd. At the end of 2 hr, Fd was added at a concentration as described above. Pulsing was continued for another 2 hr. After washing, the cells were treated as above but not co-cultured with Fd11F.

Percent suppression for both protocols was calculated using the formula:
The Ab level of the unpulsed culture was used as the background and subtracted from the levels of the Fd-pulsed and Fd11F cultures prior to the calculation of percent suppression.

**Suppression of the Serum Antibody Response using Fd11F**

DBA/2 mice were primed with C-molecule and then co-immunized with Fd and KLH 21 days later. The animals were then bled from the tail 21 days later and ranked, using ELISA, as "high" responders (>0.5) or "low" responders (<0.5) to Fd. They were divided into Fd11F, A10F, and PBS "treatment" groups with each group containing an equal number of "high" and "low" responders. Mice were then given either 10 µg Fd11F, 10 µg A10F or PBS intravenously (i.v.), allowed to recover for several hours, and boosted with Fd and KLH. They were bled seven days later and the anti-Fd and anti-KLH Ab responses were assessed as paired samples for each individual animal. Prior to their addition to the ELISA plates, serum samples were diluted at 1/100 and 1/200 using PBS-Tween. The data at 1/100 were recorded as an "X-Y" plot (see RESULTS). Similar values were obtained at both dilutions.
B16G-eluted samples and individual fractions prepared from FPLC and preparative SDS-PAGE were analyzed using 10% (w/v) polyacrylamide gels based on the method of Laemmli (1970). The mini-Protean II slab gel apparatus (Bio-Rad) was used according to the manufacturer’s instructions. Samples were concentrated by acetone precipitation and then solubilized in SDS sample buffer consisting of 10% glycerol (w/v), 2% SDS (w/v; B44244, BDH, Toronto, Ont.), 62.5 mM Tris-HCl buffer, bromophenol blue and 10 mM dithiothreitol (DTT, Bio-Rad). Prior to loading, the samples were heated to 100°C for 4 min. Between 1 to 2 μg of protein were loaded per lane. When a protein determination was not made, between 5 to 10% of the total volume of the unconcentrated sample was used for the analysis. See APPENDIX for further details about gel buffers and solutions.

Protein bands were visualized using silver staining based on the method of Wray et al. (1981). The gel was fixed in 100 ml of 50% methanol:10% glacial acetic acid for a minimum of 30 min. After the removal of fixative, the gel was rehydrated in 10% glacial acetic acid:10% methanol. The process was assisted by heating the gel in a microwave oven for 1 min. Rehydration was continued by the removal of the methanol-acetic acid mixture and replacement with distilled water. The gel was rehydrated for another 5 min at room temperature. After the water was removed, the gel was equilibrated in 100 ml of distilled water containing
33 μl of 100 mM DTT. Again, the gel was heated in a microwave oven for 1 min. The solution was replaced with 100 ml of distilled water containing 1 ml of 10% AgNO₃ (S 6506, Sigma). This was continued for 15 min at room temperature. During this time, developing reagent, consisting of 40 ml 15% Na₂CO₃ and 200 μl of 37% formaldehyde (F-79B, Fisher) in 200 ml of distilled water, was prepared. The AgNO₃ solution was poured off and the gel was rinsed with about 20 ml of distilled water. Immediately following the removal of the water, the gel was rinsed with about 20 ml of developing reagent. This was replaced with the remainder of the developing reagent. The reaction was continued until the bands began to appear. The reaction was stopped with 20 ml of 2.3 M citric acid. Lastly, gels were transferred to distilled water for storage until dried using a slab gel dryer (Bio-Rad).

High Performance Gel Filtration

B16G-eluted Fd11F was fractionated by high performance gel filtration using the Pharmacia chromatography system (FPLC). Samples were concentrated to a volume of 200 μl, filtered using a 0.22 μm filter (SLGVLO4S, Millipore, Bedford, MA) and loaded onto a Superose 12 column (HR 10/30, Pharmacia) at a flow rate of 0.25 ml/min. Between 150 to 200 μg of total protein were loaded per run. Absorbance at 280 nm was recorded using the FPLC integrator unit. Material was eluted with PBS and 48 0.5 ml fractions,
equivalent to one column volume, were collected. The column was calibrated using the following \( M_r \) markers: thyroglobulin, 669k \( M_r \); IgG, 150k \( M_r \); BSA, 66k \( M_r \); cytochrome C, 12.4k \( M_r \). Fractions were held at 4°C until assayed for Ag-binding activity. Fractions assayed for biologic activity were sterilized by filtration using a 0.22 \( \mu \)m filter (Millipore).

Preparative Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

B16G-eluted Fd11F was also fractionated using preparative SDS-PAGE. The Protean II slab gel apparatus (Bio-Rad) was used according to manufacturer’s instructions. Two hundred micrograms of Fd11F and DC10 material were solubilized in SDS sample buffer consisting of 10% glycerol (w/v), 2% SDS (w/v), 62.5 mM Tris-HCl buffer and bromophenol blue. The samples were resolved in adjacent lanes using a 12% (% T) polyacrylamide gel. See APPENDIX for further details about gel buffers and solutions.

The gels were run at constant current (35 mA) and cooled with circulating polyethylene glycol. Electrophoresis was continued until the dye front reached to within 1 cm of the end of the gel. After electrophoresis, the gel lanes were cut into 5 mm slices and each piece was minced and placed into polypropylene tubes containing 0.75 ml of 0.05 M \( \text{NH}_4\text{HCO}_3 \). After elution for 18 h at 4°C, the material was transferred to a microdialysis unit (1200 MD, BRL, Gaithersburg, MD) and dialyzed against RPMI 1640 for 18
h. A second round of elution and dialysis was carried out and the eluates were combined for both reducing SDS-PAGE analysis and Ag-binding ELISA determinations.

Two-dimensional Gel Electrophoresis (IEF x SDS-PAGE)

Instructions provided with the mini-Protean II IEF and slab gel apparatus (Bio-Rad) were followed. Modifications are listed below. Glass tubes for the first dimension were washed in detergent (RBS-35, Pierce, Rockford, IL) and rinsed with tap water followed by distilled water. The tubes were then boiled in 0.1M HCl for 30 min and rinsed with tap water, followed by distilled water and dried.

The first dimension gel solution was prepared by adding urea (B10290, BDH), HPLC-quality water, acrylamide solution, 3,[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and ampholines (pH 3.5-10, LKB, Pharmacia) together and then heating the mixture in a 37°C water bath to dissolve the urea. Any particulate matter was removed by filtering (0.45 μm, 4184, Gelman). The solution was degassed for 10 to 15 min using water suction. Immediately following the addition of ammonium persulphate (APS; Bio-Rad) and N,N,N',N'-Tetramethyl-ethylenediamine (TEMED; Bio-Rad) to the gel solution, the tube gels were casted and allowed to polymerize for 2 to 3 hr.

Prior to sample loading, degassed solutions of anolyte and catholyte were prepared. Excess unpolymerized gel was removed
from the top of the solidified gels. Any tubes with trapped air bubbles were discarded. Sample reservoirs were connected to each of the tube gels and filled with catholyte. Sample (about 10 µg in 20 µl of sample buffer) was loaded using a microsyringe by underlaying the catholyte. The sample was protected by the application of 30 µl of overlay buffer. The sample gels were focussed along with a tube gel containing 6 µl of Methyl Red marker (1mg/ml, M 5768, Sigma), and two other blank gels (used for the measurement of the pH gradient). Focusing was carried out at 500 V for 10 min and 1500 V for 90 min.

During the IEF stage, 12% SDS gels were casted. Thirty minutes before the conclusion of IEF, stacking gels (5 mm in length) were poured. Following polymerization, the wells were washed out with distilled water and Mr standards were prepared for loading. At the completion of IEF, each tube gel was extruded length-wise into the well of the second dimension SDS gel and equilibrated for 3 min in 2 x SDS sample buffer with DTT. Molecular weight standards were loaded and electrophoresis was performed as described. See APPENDIX for further details about gel buffers and solutions.

The three tube gels without samples were extruded and cut into 1 cm sections and each group of 3 x 1 cm gel pieces was placed into a tube containing 250 µl of HPLC-quality, degassed distilled water. The ampholines were eluted for 2 hr at room temperature. pH estimates were made by immersing pH test strips (pH 5-10, BDH) into each of the tubes.
RESULTS

Recovery of Fd11F from Culture Supernatants

The SN of overgrown cultures of Fd11 hybridoma containing Fd11F, were passed over a column of Sepharose CL-4B beads to which B16G was covalently linked. After extensive washing of the column with phosphate buffered saline (PBS), Fd11F was eluted using 0.1 N HCl and immediately neutralized with Tris-HCl. It was then dialyzed against PBS and concentrated, divided into aliquots and frozen at -70°C. Samples were taken from each batch for protein determination, SDS-PAGE analysis and measurement of immunoreactivity. Complete tissue culture medium including 10% FCS, or SN from the fusion parent BW5147 or DC10 hybridoma, another BW5147 fusion product of unknown specificity were used to prepare control material with a duplicate B16G-Sepharose column.

Immunochemical Properties of Fd11F

The recovery of Fd11F from the immunoabsorbent columns could be monitored using a solid-phase ELISA. Various concentrations of Fd11F and DC10 control material were adsorbed to ELISA wells overnight. After washing, B16G and an irrelevant mAb of the same subclass (IgG2a) were added and developed with rabbit anti-mouse immunoglobulin-alkaline phosphatase (RaMIg-AP). Fig. 1 shows that when solid-phase material was adsorbed at a
Fig. 1. Binding of B16G to Fd11F adsorbed to polystyrene wells.

Fd11F and DC10 material were adsorbed to ELISA wells and detected with B16G, used at 18 μg/ml. The ELISA was developed with RaMlg-AP, used at 0.5 μg/ml. The irrelevant mAb, CAMAL-1, used at the same concentration as B16G, bound to neither Fd11F or DC10 material.

(●) Fd11F + B16G; (●) Fd11F + CAMAL-1; (○) DC10 + B16G; (□) DC10 + CAMAL-1.
concentration of 6.6 µg/ml, B16G reacted strongly with solid-phase FdllF but not with DC10 material. The irrelevant mAb, CAMAL-1, did not bind to either the FdllF or DC10 layer. Representative data of three experiments are shown.

The ELISA was also used to test the binding of FdllF to solid-phase Fd. In this assay system, various concentrations of FdllF were added to Fd-coated plates. The bound FdllF was then detected with B16G and RaMIg-AP. Fig. 2A shows a typical curve where various concentrations of FdllF were added to Fd-coated plates. Evidently, B16G could still react with FdllF in spite of its binding to Fd. The negative control of Fig. 2A is the medium-derived B16G eluate prepared concurrently with FdllF. Essentially no reactivity from the control preparation was observed. Fig. 2B shows that eluted control material from cellular SN (DC10 SN) also did not show any reactivity in the assay. Thus for any given FdllF preparation, its reactivity in the "sandwich" ELISA suggested that both its Ag-binding site and B16G-binding site were isolated intact.

Fig. 3 is a control experiment where an irrelevant mAb was compared to B16G. B16G and CAMAL-1 were used at equivalent concentrations of 18 µg/ml to detect FdllF. It can be seen that nonspecific binding of CAMAL-1 for FdllF began only when high concentrations of material were employed (ie. > 4 µg/ml). However, even at these concentrations, the nonspecific signal was exceeded by the reaction of B16G and FdllF. Representative results from three different experiments are shown.
Fig. 2. Binding of Fd11F to Fd adsorbed to polystyrene wells and its detection with B16G.

Fd was adsorbed to ELISA wells at 1 μg/ml. Materials eluted from B16G affinity columns were added to solid-phase Fd at various concentrations and then detected with B16G and RaMIG-AP, used at the same concentrations as for Fig. 1.

A. Fd11F (●) compared to medium control (○). Representative results from seven different preparations are shown.

B. Fd11F (●) compared to the cellular SN control DC10 (○). Results (mean ± SEM) from 3 separate preparations are shown.
Fig. 3. The reactivity of B16G and the irrelevant mAb, CAMAL-1, with Fd11F.

Fd11F and DC10 material were added to Fd-coated wells at various concentrations. They were detected with either B16G or CAMAL-1, used at 18 μg/ml, and RaMIG-AP.

(*) B16G + DC10; (•) B16G + Fd11F; (○) CAMAL-1 + DC10;
(□) CAMAL-1 + Fd11F.
Fd11F showed selectivity for Fd in binding when compared to other Ag. The data presented in Table I demonstrate that when Fd, serum albumin, and another iron-containing protein, cytochrome C were adsorbed to separate wells, Fd11F bound to Fd only. None of the other B16G eluates showed any binding preference for the Ag that were used.

The binding of Fd11F to Fd in solution was also shown. Various concentrations of Fd were incubated with a constant amount of Fd11F. The mixture was then added to Fd-coated wells and the amount of free Fd11F was measured as before in the "sandwich" ELISA. Fig. 4 shows that increasing concentrations of Fd could inhibit the amount of Fd11F binding to Fd-coated plates. Cytochrome C, used as the control protein, did not bind to Fd11F, and consequently did not inhibit Fd11F binding. Representative data of three experiments are shown.

The fine specificity of Fd11F binding was examined using this inhibition assay and analogs to Fd. Analogs, expressing either the N-determinant or the C-determinant, and a third analog, OFd, were mixed at various concentrations with a constant amount of Fd11F. The amount of unbound Fd11F was then be measured using the "sandwich" ELISA.

Prior to performing this assay, the antigenicity of the analogs was confirmed using Fd determinant-specific mAb. The analogs were adsorbed to ELISA wells and then incubated with the mAb specific for the amino-terminal and carboxyl-terminal determinants. Fig. 5 shows that the amino-terminal determinant
Table I. The binding of Fd11F to Fd.

Fd or other antigens were coated onto ELISA wells at 1 µg/ml. Fd11F or control materials were added at 4 µg/ml and detected with B16G and RaMIg-AP. Representative data of three different experiments are shown.

HSA: human serum albumin

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<th>Source of B16G eluate</th>
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<tr>
<td></td>
<td>Fd</td>
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<tr>
<td>Fd11</td>
<td>.567</td>
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<tr>
<td>medium</td>
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<td>DC10</td>
<td>.050</td>
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<td>BW5147</td>
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Fig. 4. The binding of soluble Fd to Fd11F in fluid-phase.

Fd11F was mixed with various concentrations of Fd (*) or cytochrome C (○) prior its addition to Fd-coated wells. Fd11F was detected as for Fig. 2.
Fig. 5. Antigenic analysis of Fd and its analogs using determinant-specific mAb.

N-molecule (N), C-molecule (C), OFd, Fd, KLH and BSA were coated onto ELISA wells and then incubated with the Fd-specific mAb, 16a and 6-90, both used at 2 μg/ml. 16a is specific for C-determinant, the carboxyl-terminal epitope of Fd and 6-90 is specific for N-determinant, the amino-terminal epitope of Fd. Monoclonal antibodies were developed with RaMlg-AP.
specific mAb, 6-90, bound to both the carboxypeptidase A-treated analog (N-molecule) and Fd. It did not bind to the trypsin-treated analog, known as C-molecule or the irrelevant Ag, KLH or bovine serum albumin (BSA). In contrast, the carboxyl-terminal determinant-specific mAb, 16a bound to C-molecule and Fd; there was a slight degree of binding to N-molecule (approximately 10% of the C-molecule signal). This probably indicated that carboxypeptidase A digestion was not complete and a small percentage of the N-molecule preparation was contaminated with the undigested Ag. Incomplete digestion of N-molecule has been noted previously in this laboratory. 16a did not bind to either KLH or BSA. OFd was reactive with both 6-90 and 16a. Typical results of seven different experiments are shown.

The fine specificity of FdllF binding was subsequently determined using the analogs. Fig. 6 shows that C-molecule was the most effective inhibitor of FdllF binding causing 50% inhibition at .312 µg/ml. Fd was the next best inhibitor followed by OFd. More than 15 times the amount of N-molecule was required to achieve the level of inhibition observed with C-molecule and Fd. In fact, the amount of N-molecule that was required suggested that inhibition was most likely due to the contamination of the preparation with undigested Fd. The data indicate that FdllF is specific for the C-determinant of Fd. Representative data from three different experiments are shown.

In summary, FdllF was shown to bind to both Fd and the mAb, B16G which allowed its quantification, at least at the
Fig. 6. Inhibition of FdllF binding by Fd and its analogs.

FdllF was mixed with various concentrations of N-molecule (○), C-molecule (•), OFd (●), Fd (□), or cytochrome C (△) prior to its addition to Fd-coated wells. FdllF was detected as in Fig. 2.
immunochemical level. Fd11F was shown to bind preferentially to Fd and through the use of analogs it was determined to be specific for the carboxyl-terminal region of Fd. The next set of studies examined some of the biological properties of Fd11F.

**Biological Properties of Fd11F**

Development of an antibody culture assay for the measurement of suppression

Having established the immunochemical properties of Fd11F, it was of interest to examine some of its biologic properties. To study the effect of Fd11F on the Ab response, an in vitro Ab production assay was developed.

Several different protocols were tried before one was chosen. Initially, the C-molecule-primed animals were immunized twice with Fd before the harvesting of spleens to insure that the cells would synthesize enough Ab in vitro. In addition, it was found that the cells had to be pulsed with Ag just prior to culturing in order to provide enough stimulus for Ab production in vitro. During the pulsing period, a sufficient amount of Fd is pinocytosed and "processed" by splenic antigen-presenting cells to drive the Ab response despite the lack of soluble Ag in culture. Typical results (of six different culture experiments) of this immunization and pulsing protocol are seen in Fig. 7. It can be seen that Ab was easily detected in the SN from day 5
Fig. 7. Detection of anti-Fd Ab in the SN of spleen cell cultures prepared from B10.D2 mice that were immunized with C-molecule and Fd twice.

Spleen cells were incubated with either Fd or medium for 2 h before culturing. SN were collected five, six, and seven days after initiation of culture. Ab content of SN was measured using ELISA.
cultures using ELISA. In fact, Ab production continued through to day 6 and day 7. A culture containing medium pulsed cells served as background control for the ELISA measurement. Once it was established that Ab could be produced in vitro, experiments were done to maximize the amount of suppression. In one modification, hyperimmune animals were treated with FdllF in vivo before their spleens were removed for culturing. This was thought to make the cells more "susceptible" to the in vitro effects of FdllF. Preliminary experiments were also tried where the spleen cells were pulsed concurrently with Fd and FdllF.

Eventually, one method was selected. Spleen cells were prepared from Fd-immune B10.D2 mice that were previously immunized with C-molecule (to establish immunity) and boosted with Fd once. The cells were pulsed with Fd, washed free of soluble Ag, and cultured with either control material, FdllF-containing material or medium for seven days. SN were collected and the anti-Fd Ab levels were measured using ELISA. A comparison of the levels served as a measure of suppression. Substrate development was allowed until the maximal absorbance on the plate reached approximately one.

A curve plotting the relationship between absorbance produced and the concentration of Ab that bound to Fd-coated wells is shown in Fig. 8. Mouse Ab eluted from a Fd-Sepharose column was used as a source of affinity-purified material. The curve was approximately linear from 0.1 to 1.0 absorbance units. Therefore it could be assumed that absorbance would be directly
Fig. 8. Relationship between Absorbance at 405 nm vs. concentration of anti-Fd Ab.

Affinity-purified anti-Fd Ab was incubated on Fd- and KLH-coated plates. Ab was detected using RaMIG-AP. Reaction was developed for 2 h.

Fd: (•)  KLH: (○)
related to the concentration of Ab that was actually present in the SN. The specificity of the affinity-purified Ab for Fd was shown by its lack of reactivity with KLH. Representative data of three experiments are shown.

The specificity of the anti-Fd Ab secreted into the SN for the Fd analogs and unrelated Ag was then determined. In Fig. 9A it can be seen that essentially all of the Ab was specific for the amino-terminal determinant. The relative lack of Ab directed to the C-molecule was consistent with the finding that C-molecule immunization of B10.D2 animals induces little Ab directed to that determinant (data not shown). The SN did not react with the irrelevant Ag, KLH and BSA. For comparison, the serum Ab activity of Fd-immune B10.D2 animals is shown in Fig. 9B. The serum activity is quite similar to that of the SN. The apparent increase of Ab reactive with N-molecule over Fd was probably due to differences in binding of Fd or N-molecule to ELISA plates or some nonspecific binding of serum Ab. Indeed, the nonspecific binding activity of the serum, as shown by its reactivity with KLH, is greater than that of the SN (c.f. Fig. 9A).

Correlation between bioactivity and antigen-binding activity of Fd11F

Fig. 10 is the "dose-response" curve of Fd11F correlating suppressive activity in vitro (ie. the response) with Ag-binding
Fig. 9A. Specificity of anti-Fd Ab from Fd-immune spleen cell cultures for Fd analogs.

SN from seven day cultures were harvested and Ab level determined using ELISA. Typical results of six different cultures are shown.

N: N-molecule   C: C-molecule

Fig. 9B. Specificity of serum anti-Fd Ab from Fd-immune B10.D2 mice for Fd analogs.

Ab level at a 1/400 dilution of serum was determined using ELISA. Results (mean ± SEM) from three separate serum samples are shown.

N: N-molecule   C: C-molecule
Suppressive activity is expressed as percent suppression. Percent suppression was calculated by comparing Ab levels of cultures containing Fd11F to the levels measured in the control culture. Ag-binding activity is expressed as the sandwich ELISA value of the Fd11F lot tested. The pooled data from seven lots are shown. Lots 1 to 5 were assayed at two or three concentrations while lots 6 and 7 were assayed at only one concentration. Following its elution from B16G affinity columns, each lot was measured for protein content, Ag-binding activity, and bioactivity. A negative control sample was prepared concurrently with each Fd11F lot. This sample was used as the respective negative control for the determination of net Ag-binding activity and bioactivity.
activity as measured by "sandwich" ELISA (i.e. the dose).
Response values were expressed as percent suppression (calculated using the Ab level of the culture containing Fd11F as compared to the untreated culture). Dose values were expressed as Ag-binding activity, rather than total protein concentration to allow comparison of the data from different batches of Fd11F.

The curve is a compilation of results from seven different lots of Fd11F. The curvilinear relationship between bioactivity and Ag-binding activity suggest that the two activities are related and can be ascribed to Fd11F. The data representing lots 4 and 7 did not conform with the majority of observations. The reason(s) for this is unknown. One possibility is that the total Ab levels produced by the untreated, Fd-pulsed cultures in these experiments were comparatively low. This resulted in a disproportionately high percentage of suppression. It was also observed that the suppression produced by Fd11F did not exceed 60%. Almost complete suppression was eventually achieved following a revision to the culturing protocol (see below, Fig. 12).

None of the cultures containing Fd11F showed an increase in Ab production as compared to untreated controls. B16G eluates made with negative control SN were prepared in parallel with each lot of Fd11F. Of the negative control preparations, only one lot (lot 4) of the seven demonstrated any suppressive activity. For these cultures, the decrease was corrected for the effect of the negative control material.
FdllF-mediated suppression was also shown to be specific for the anti-Fd Ab response. Fig. 11 shows that when FdllF, at three different concentrations, was added to lysozyme-immune cells, concentration-dependent suppression was not observed. However, this same preparation of FdllF did suppress the anti-Fd Ab response. BW5147 control material, which did not possess Ag-binding activity, is shown for comparison. It also did not demonstrate suppressive activity and was representative of the response generated by the other control materials (ie. medium and DC10 SN). Representative data of three experiments are shown.

Additional work, addressing the in vitro "specificity" of FdllF bioactivity was done. In brief, another product derived from the T cell hybridoma, A10, is suppressive for the CTL response to the murine mastocytoma, P815 (Steele, Stammers, and Levy 1985). The material, A10F, is recovered from the culture SN by affinity chromatography using B16G-Sepharose. When A10F was added to CTL cultures prepared from P815-primed mice, there was a decrease in specific cell lysis as compared to cultures that contained FdllF. By contrast, when FdllF was added to cultures of Fd-immune spleen cells, there was a decrease in Ab levels of the cultures as compared to the cultures that contained A10F. That is, FdllF was only suppressive when used in the Ab culture assay and A10F was only active when used with the CTL assay. The results from two of these "criss-cross" experiments are shown in Table II.
Fig. 11. Specificity of the suppressive activity of Fd11F.

A single preparation of Fd11F, used at three different concentrations, was added to cultures of lysozyme-immune cells and Fd-immune cells.

Anti-lysozyme Ab levels of cultures containing Fd11F (•) were compared with the untreated control culture. The percent suppression is shown. Also included is the response obtained with BW5147 control material (○) when added to the lysozyme-immune cells. It did not possess any Ag-binding activity and was added to the cultures at the protein concentrations that are indicated. To demonstrate that the same preparation of Fd11F was active, it was added to Fd-immune cells. The percent suppression of these cultures, at three concentrations, are shown (●).
Table II. Comparison of the suppressive capability of Fd11F and A10F in two assays of cellular function.

Fd11F and A10F were each added to cultures containing either P815-specific CTL or Fd-immune spleen cells. For the P815 CTL assay, data are expressed as percent lysis and percent suppression for the culture. Percent suppression was calculated by comparing percent lysis of the cultures containing affinity-enriched material to the untreated control culture. For the anti-Fd Ab assay, data are expressed as Ab level of the culture (Absorbance 405 nm) and percent suppression. Percent suppression was calculated by comparing the Ab level of cultures containing affinity-enriched material to the untreated control culture. The results from two separate experiments are shown. Ab culture measurements were obtained using the "revised" protocol (see MATERIALS AND METHODS).

A: material added at 1 µg/ml  B: material added at 1.5 µg/ml

<table>
<thead>
<tr>
<th>Material</th>
<th>Assay</th>
<th>P815 CTL</th>
<th>Anti-Fd Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% lysis</td>
<td>% suppression</td>
</tr>
<tr>
<td>Expt. A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no factor</td>
<td></td>
<td>24.5</td>
<td>-</td>
</tr>
<tr>
<td>A10F</td>
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<td>100</td>
</tr>
<tr>
<td>Fd11F</td>
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<td>10.7</td>
</tr>
<tr>
<td>Expt. B</td>
<td></td>
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<td>-</td>
</tr>
<tr>
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<td>5.6</td>
<td>62.4</td>
</tr>
<tr>
<td>Fd11F</td>
<td></td>
<td>14.5</td>
<td>3.3</td>
</tr>
</tbody>
</table>
The "dose-response" curve of Fig. 10 indicated that the amount of suppression achieved was dependent on the Ag-binding activity of the sample. However, the maximal level of suppression that was ever attained with the assay did not exceed 60%. Whether this was a limitation of Fd11F or of the assay itself was unknown. This original assay used Fd-immune spleen cells that were pulsed for 2 h with Fd to allow for Ag processing. The cells were then washed to remove soluble Fd and then co-cultured with Fd11F (or control material) for seven days. At the end of the incubation period, the culture SN were assessed for anti-Fd Ab using ELISA.

Further development work showed that modifications to the Ab culture protocol yielded suppressive effects by Fd11F, which approached 100%. The changes to the protocol included pulsing the Fd-immune cells first with Fd11F and then introducing Fd 2 h later. After another 2 h, the cells were washed, plated and incubated for seven days without receiving any additions of factor. The day 7 SN were assessed as the in the original protocol. Representative data (of three different experiments) in Fig. 12 show that the suppressive effect was concentration-dependent and reached a maximum of 85%. BW5147 control material was not suppressive at any concentration.

Suppression of anti-Fd antibody levels in vivo

The following experiment was designed to test the effect of
Fig. 12. Suppressive activity of affinity-enriched Fd11F compared to BW5147 control material using the "revised" protocol.

Various concentrations of either BW5147 or Fd11F material were first added to Fd-immune spleen cells followed by the in vitro pulsing with Fd. Percent suppression was determined as for Fig. 10.

(•), Fd-immune cells pulsed with Fd11F; (○), Fd-immune cells pulsed with BW5147 control material.
Fd11F on the anti-Fd Ab response in vivo, using another strain of H-2d animals, DBA/2 mice.

DBA/2 mice were primed with C-molecule followed by an immunization with Fd. The mice were also immunized with the control Ag, KLH at this time. All immunizations were made subcutaneously (s.c.) using complete Freund’s adjuvant. The animals were bled 21 days later and ranked according to the magnitude of their response to Fd as measured using ELISA. They were designated as low or high responders and then divided into three groups such that each one had an approximately equal number of low and high responders. The animals within each group received either 10 μg Fd11F, 10 μg A10F or PBS i.v., were allowed to recover for several hours and then boosted with Fd and KLH, s.c.. The animals were bled seven days later and the anti-Fd and anti-KLH Ab responses before and after treatment were assessed as paired samples for each individual animal.

The data are presented as an X-Y plot where the pretreatment serum Ab level is plotted on the x axis and the post treatment serum Ab level is indicated on the y-axis. A decrease in Ab response following treatment appears as data below the "no change" line. Each data point represents one animal. Fig. 13 shows that five out of six animals that received Fd11F at the time of Ag boost had a decrease in circulating anti-Fd Ab seven days post treatment. In contrast, as shown in Fig. 14, the majority of animals from both groups of animals receiving A10F or PBS showed an increased Ab response. The X-Y plot shows the
Fig. 13. X-Y plot showing anti-Fd Ab levels of animals before and after treatment with Fd11F and Ag.

Animals were immunized with C-molecule and then co-immunized with Fd and KLH. Once immunity was established, they were boosted with Fd and KLH and given Fd11F, PBS or control material, i.v.. Matched pairs of sera were obtained from the individual animals that were bled before and after treatment. Symbols represent the x-y coordinates of pretreatment and post treatment serum Ab levels for individual animals. A decrease in Ab level is plotted as a symbol below the "no change line".

Animals were treated with either Fd11F (●), A10F (•) or PBS (□).
Fig. 14. X-Y plot showing anti-KLH Ab levels of animals before and after treatment with Fd11F and Ag.

See Fig. 13 for explanation.

Animals were treated with either Fd11F (●), A10F (●) or PBS (□).
results from the same animals, only this time the anti-KLH Ab response is plotted. The large majority of animals showed an increase in anti-KLH Ab whether treated with AlOF or Fd11F. Taken together, the data reveal that Fd11F appears to have specificity for the anti-Fd Ab response when compared to B16G-eluted control material and another Ab response to an irrelevant Ag. (B16G-reactive material was tested in vivo, four times. Representative results are shown).

To summarize, the biologic activity of Fd11F was demonstrated both in vitro and in vivo. A spleen cell assay was developed and used to show that Fd11F suppressed the Ab response in a concentration-dependent manner. Data from other experiments suggested that the suppressive effect was Ag-specific. In vivo, Fd11F treatment suppressed the serum Ab response of Fd-immune H-2d mice following Ag challenge. As controls, AlOF and PBS had no effect on the development of the secondary response. The anti-KLH Ab response was not affected by any of the treatments.

With the availability of both an immunochemical and biological means of measuring Fd11F, biochemical analysis of the Fd11F Ag-binding molecule(s) was now possible.

Biochemical Analysis of Fd11F

Introduction

When the eluates from B16G columns were analyzed using SDS-
PAGE, run under reducing conditions, a characteristic and reproducible banding pattern was observed with silver staining. Fig. 15 shows the result from a representative Fdl1F recovery experiment. Equal amounts of protein (1 to 2 μg) were loaded into each well. Affinity chromatography yielded only partially purified preparations of Fdl1F since bands resembling BSA and probably other serum components co-eluted with Fdl1F. Two other predominant bands always appeared. Of primary interest was a unique band at approximately 30k $M_r$. In addition, a band at approximately 80k $M_r$ was routinely recovered. Lesser amounts of 80k $M_r$ material also appeared in the lane containing the sample derived from non-Fdl1 SN, whether it was prepared from medium, BW5147 or DC10 SN. (Note: Subsequent references in the text to the "80k" or "30k" $M_r$ proteins refer to the protein at approximately 80k $M_r$ and the protein at approximately 30k $M_r$ of Fig. 15, respectively.)

From this starting point, efforts were directed at the biochemical identification of the Ag-binding moiety of Fdl1F. The aim was to further resolve the Fdl1F-enriched material from the B16G-Sepharose column and identify the molecule(s) by both their Ag-binding and biologic activities.

Analysis of Fdl1F using high performance gel filtration: correlation between antigen-binding and suppressive activity

High performance gel filtration (FPLC, Pharmacia,
Fig. 15. SDS-PAGE of eluates from B16G affinity columns.

Eluates derived from Fd11 SN (A) or medium containing 10% FCS (B) were analyzed using a 10% SDS gel run under reducing conditions. Position of Mᵦ markers are indicated. Actual Mᵦ equals number shown multiplied by 1000. The predominant bands at 80k and 30k Mᵦ were consistently obtained with Fd11 SN. Bands were developed by silver staining. Typical results of six different preparations of Fd11F (and control material) are shown.
Superose 12 column) was the first separation method chosen since it allowed fast recovery of the native molecules without the use of denaturing conditions or buffers which would cause dissociation of non-covalently associated materials.

The starting materials were the eluates from the B16G-Sepharose columns. Control material was chromatographed first to avoid cross-contamination of proteins. After the column was washed with eluent, Fd11F material was loaded.

Each fraction was assayed for Ag-binding activity using the "sandwich" ELISA and analyzed by SDS-PAGE run under reducing conditions. Fig. 16 shows the FPLC protein profiles of medium, BW5147, and Fd11F material. Equal amounts of protein were loaded for each sample. A large protein peak beginning at the void volume was recovered from the Fd11F sample (The void volume of the column was calculated based on experimental data and the performance specifications provided by Pharmacia). This large amount was spread over several fractions (fractions 16 to 22) as suggested by the Ag-binding activity profile, shown in Fig. 17. (Representative results of three determinations are shown.) The peak of immunoreactivity (fraction 17) coincided exactly with the presence of a 80k and 30k $M_r$ band identified in the fraction as revealed by SDS-PAGE (Fig. 18C). Fractions 16 to 19 are shown. In addition, two 45k $M_r$ bands unique to the Fd11F sample were present in fraction 16. They did not appear to contribute to Ag-binding activity. Lesser quantities of the 80k $M_r$ material appeared in both control lanes, consistent with
Fig. 16. Protein profiles (280 nm) of B16G eluates derived from medium (A), BW5147 (B), and Fd11F (C) SN fractionated on a Superose 12 column using FPLC.

Enhanced recovery of material at the void volume derived from Fd11F SN is shown. Equivalent amounts of protein (188 µg) were loaded and chromatographed according to the conditions described in Materials and Methods. Relative molecular mass markers are indicated. Markers included thyroglobulin (TG), 669k M_r; IgG, 150k M_r; BSA, 66k M_r; and cytochrome C, 12.4k M_r. In total, 48 fractions (0.5 ml) were collected. Protein traces are for fractions 15 to 47. Typical data of five different fractionations are shown.
Ag-binding activity of FPLC fractions obtained from Fd11F (•), BW5147 (□), and medium (○) elution profiles of Fig. 16.

Ag-binding activity was measured as for Fig. 2. One hundred microlitres of each fraction were added to Fd-coated plates and developed with B16G. ELISA values are the mean of duplicate determinations. A preliminary FPLC experiment had shown that Ag-binding activity was not detectable in the region of fractions 35 to 48. Representative results of three different determinations are shown.
Fig. 18. SDS-PAGE analysis of FPLC fractions (shown in Fig. 17) 16 to 19 inclusive.

Ten percent SDS gels containing reducing agent were developed by silver staining. Twenty-five microlitres from each fraction were concentrated and electrophoresed. Position of Mₐ markers are indicated. Actual Mₐ equals number shown multiplied by 1000. Representative data of three analyses are shown.

A: medium control    B: BW5147 material    C: Fd11F
previous results. There were no other visible bands unique to Fd11F (or to the control samples) in the remainder of the fractions that were analyzed.

A second FPLC experiment was conducted using a fresh set of BW5147 and Fd11F eluates with the intent of collecting fractions for bioassay. Fractions 15 to 20 from each sample were selected for testing. Table III shows the percent suppression of Ab production by spleen cells that were co-cultured with the fractionated material. It can be seen that maximal suppression occurred with fraction 18 from the Fd11F sample. Therefore material from this region of the fractionation profile demonstrated suppressive activity.

Thus, Ag-binding (Fig. 17) and suppressive (Table III) material were recovered from the void volume of the gel filtration column. However, when the fractions were analyzed by polyacrylamide-SDS gels run with reducing agent, a unique band of 30 kDa was recovered along with an 80 kDa protein. Additional bands at 45 kDa, not directly related to Ag-binding, were also seen. Together, these data suggest that the Ag-binding, suppressive material eluting from B16G is composed of a high Mr moiety made up of a number of identifiable peptides with Mr between 30k and 80k.

Identification of the antigen-binding molecule using SDS-PAGE

Although FPLC fractionation provided more evidence relating
Table III. Ability of various fractions eluting from FPLC Superose 12 column to inhibit the in vitro Ab response of Fd-immune spleen cells. Percent suppression was calculated by comparing levels of Ab formation in cultures incubated with eluted materials to levels detected in untreated controls. A total of 475 μl from each fraction were added to the cultures. Cultures containing the fractionated material were incubated for seven days. Ab culture measurements were obtained using the "original" protocol (see MATERIALS AND METHODS). Representative results of three different culturing experiments are shown.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Percent suppression</th>
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<tbody>
<tr>
<td></td>
<td>BW5147</td>
</tr>
<tr>
<td>15</td>
<td>-8</td>
</tr>
<tr>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>17</td>
<td>2</td>
</tr>
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<td>19</td>
<td>13</td>
</tr>
<tr>
<td>20</td>
<td>-10</td>
</tr>
</tbody>
</table>
Ag-binding activity and bioactivity with Fd11F, it did not indicate which molecule(s) was actually responsible for Ag-binding.

Another attempt to identify the Ag-binding moiety was made using preparative SDS-PAGE (run without reducing reagent). B16G eluates of Fd11F and DC10 SN were electrophoresed on a 12% slab gel. The gel was cut into equal-size strips and the material eluted from the individual slices was analyzed by SDS-PAGE run under reducing conditions and assayed for Ag-binding activity. The Ag-binding profile is shown in Fig. 19. Major regions of activity were located at slices 12 to 14 and at the end of the gel (slices 20 to 22). DC10 eluted material did not have any Ag-binding activity. The activity of slices 12 to 14 coincided exactly with the presence of bands in the 30k $M_r$ region that were unique to the Fd11F sample as assessed by reducing SDS-PAGE. The gels actually resolved a doublet in this region. This is shown in Fig. 20. No Ag-binding activity was detected with the 80k $M_r$ bands that resolved at slice 4 of Fd11F material (see Fig. 20). In fact, no other distinct bands unique to Fd11F were detected. Two faint bands at 68k $M_r$ were visible in lanes 13 and 14, for both "F" and "D" of Fig. 20. Whether they were protein or a chemical artifact was unknown. Their appearance in a control lane not loaded with a protein sample ("C") suggested that they were silver staining artifacts.

The protein bands eluted from slices 20 to 22 of the DC10 or Fd11F samples, if actually present, were not visible; most likely
Fig. 19. Ag-binding activity of DC10 and Fd11F material eluted from slices of a 12% preparative SDS-PAGE gel run under non-reducing conditions.

Two hundred micrograms each of affinity-enriched Fd11F (•) or DC10 (○) material were fractionated using preparative SDS-PAGE under non-reducing conditions. Recovered material from individual slices was tested for Ag-binding activity as in Fig. 2.
Fig. 20. PAGE of material from selected fractions of Fig. 19 using a 10% SDS gel run under reducing conditions.

Fraction 4 shows the 80k $M_r$ protein recovered from both Fdl1F and DC10 control material. Fractions 12 to 14, which exhibited Ag-binding activity (Fig. 19), show bands unique to Fdl1F from the 30k $M_r$ region.

F: Fdl1F  D: DC10 material

C: control lane showing silver staining "artifact"
due to the inability of the gel to resolve low $M_r$ proteins (ie. $< 14k M_r$). Whether the material responsible for the ELISA activity was a breakdown product of the 30k $M_r$ species or represents a different protein that binds to both Fd and B16G is unknown. Further work would be required to clarify its relationship, if any, to the 30k $M_r$ protein.

It should be noted that three separate preparative SDS-PAGE fractionation experiments were performed with the aim of recovering bioactivity from gel slices, without success. It was possible that the residual amounts of SDS in the eluted fraction material had a detrimental effect on the growth of the Ab cultures. An alternative explanation was that SDS-PAGE fractionation had destroyed bioactivity by the separation of two or more non-covalently associated molecules, one of which was Ag-binding. On occasion, other bands unique to the FdllF sample (eg. Fig. 18C) have been recovered along with the 30k $M_r$ molecule.

The appearance of the 30k $M_r$ protein in both the FPLC fractions and the material eluted from the non-reducing preparative SDS-PAGE fractions strongly suggested that it was the moiety responsible for the Ag-binding activity. However, to determine if the 80k $M_r$ molecule was actually unique to FdllF, it was subjected to two-dimensional gel electrophoresis. The remainder of the selected fractions from the FPLC experiment of Fig. 18 were used. Material from fraction 17 of the FdllF sample and pooled material from fractions 17 to 19 of the medium sample
were analyzed in parallel. Fig. 21 shows that, within the limits of the technique, the 80k $M_r$ proteins from medium and Fd11F samples were not only of the same $M_r$, but had the same isoelectric point as well. It is generally assumed that two different proteins would not have the identical pI and $M_r$. Therefore in spite of the preponderance of the 80k $M_r$ protein of Fd11F over control material, the two proteins were probably identical and were derived not from the Fd11 hybridoma, but the FCS of the complete medium mixture.
Fig. 21. Two-dimensional gel electrophoresis of the 80k Mr proteins of medium and Fd11F.

Equal amounts of 80k Mr material from both medium (panel A) and Fd11F (panel B) were separated by IEF using pH 3-10 ampholytes and reducing SDS-PAGE. After focussing, the tube gels were layered on top of 12% SDS-PAGE gels and incubated in sample buffer containing reducing agent for 5 min. Following incubation, electrophoresis in the second dimension was begun concurrently for both samples. Gels were fixed and silver-stained. Isoelectric point and Mr calibrations are indicated.
It has been reported that Fd11F, produced by the Ts hybridoma, Fd11, was recovered using the TsF-specific mAb, B16G. Fd11F possessed both Ag-binding activity and the ability to suppress the anti-Fd Ab response in vivo (Steele, Chu, Chan, North, and Levy 1987). In this thesis, Fd11F was characterized further at the immunochemical, biological, and biochemical levels. It was shown that Fd11F bound to and demonstrated selectivity for solid-phase Fd. The factor reacted also with fluid-phase Ag. This property was used to determine the fine specificity of Fd11F binding. The biological effect of Fd11F on the anti-Fd Ab response was measured using a spleen cell culture system. With this assay, Fd11F was shown to suppress Ab production in a concentration-dependent manner. Furthermore, additional experiments suggested that the suppressive effect was Ag-specific. Biochemical characterization was aimed at isolating the Ag-binding moiety of Fd11F. By using gel filtration, both Ag-binding and biologic activity were recovered from a high $M_r$ peak ie. $> 500,000$. When non-reducing preparative SDS-PAGE was used, a single Fd11-associated, 30k $M_r$ polypeptide, which appeared to bind Ag, was isolated. These findings are discussed below.
Isolation of Fd11F was possible because of the TsF-specific mAb, B16G. As mentioned in the INTRODUCTION, B16G was initially developed to study the P815 tumor system. The Ab itself was shown to be active, both in vitro and in vivo. It was then used to isolated A10F, a TsF which suppressed the CTL response to P815. B16G has been employed as a tool to study suppression in other Ag systems. Steele et al. (1987) showed that B16G recognized TsF₁, but not TsF₂ or TsF₃ of the NP system. This indicated that B16G was not only useful outside the systems in which it was developed, but that B16G was "class-specific" with respect to recognizing different types of TsF. In a completely different experimental system, B16G was used to study UV radiation-induced suppression. It is known that exposure of mice to UV radiation can lead to an impairment in immune function. Adoptively transferred lymphoid cells from UV-treated animals fail to reject a UV-induced syngeneic tumor as compared to untreated controls. Failure of rejection was attributed to the induction of tumor-specific Ts (Fisher and Kripke 1977). An association between defective Ag presentation and systemic suppression of the CS response was noted in mice following exposure to UV radiation (Noonan et al. 1981). B16G was used to show that TsF as well, was implicated in the UV-induced systemic suppression of the CS response (Yee et al. 1989). In vivo, B16G abrogated the effect of UV radiation on the CS response. The
investigators also prepared B16G immunoadsorbents and recovered suppressive material from Ts culture SN. Lastly, Sehon and colleagues have used B16G for the isolation of suppressive material involved in the regulation of the IgE response (Sehon et al. 1989). Thus, B16G has been effective in the study of suppression in several laboratories. It has a modulating effect on the immune response when it is administered in vivo and can be used for the recovery of suppressive molecules in different Ag systems.

The use of Ab reagents specific for a constant region of the TsF molecule is not exclusive to B16G. In an attempt to identify putative TCR isotypes, Tokuhisa and Taniguchi (1982) raised an alloantiserum specific for the murine immunoglobulin heavy chain. The antiserum was found to bind TsF. Using the reagent, they went on to identify allotypic determinants on the constant region of the Ag-binding chain of a KLH-TsF.

Sorensen and Pierce (1985) prepared mAb that were either specific for inducer or effector TsF. Although they produced a battery of mAb, none of them were clonotypic i.e. none were reactive with the Ag-binding, variable regions of any of the TsF, which were used as immunogens. Similarly, Iverson and colleagues isolated mAb that were specific for the Ag-binding chain of inducer and effector TsF, respectively (Ferguson, Beaman, and Iverson 1985; Ferguson and Iverson 1986). Both groups used the mAb in the recovery of TsF for functional and biochemical analysis.
Immunochemical Properties of Fd11F

Direct binding of Fd11F to Fd was shown using ELISA. Initially, however, the data of Fig. 1 showed direct and specific binding of B16G to Fd11F-coated plates. With a variation in the immunoassay, Ag-binding by Fd11F was demonstrated as well. That is, Fd11F, but not material prepared from medium containing FCS or from the irrelevant hybridoma, DC10, bound to Fd-coated plates. The data of Table I, which showed binding of Fd11F to Fd, but not to two other unrelated Ag, indicated that Fd11F reactivity was not explained by nonspecific adsorption to a protein-coated surface.

The possibility that the binding was due to a particular, solid-phase conformation of Fd was tested by determining whether Fd11F could react with Fd in solution. It was found that various concentrations of Ag could inhibit the binding of Fd11F to solid-phase Fd. As shown in Fig. 6, the level of inhibition approached 100% with Fd and C-molecule. This verified that Fd11F binding was identical whether Fd was in fluid- or solid-phase and was independent of any "fixed" conformation of Fd. The inhibition ELISA allowed the binding of Fd11F to be quantified as well. It was shown in Fig. 6 that Fd11F was specific for the C-determinant of Fd. The observation that C-molecule and Fd were better inhibitors than O/Fd (0.312 µg/ml vs. 1.25 µg/ml for 50% inhibition) suggested that Fd11F binding to Fd (or its analogs) was not simply due to charge effects. Since Fd is an acidic
protein (isoelectric point of Fd is 3.7), it was conceivable that binding was due to the net negative charge of Fd while in solution. If so, then OFd would be expected to be a better inhibitor of Fd11F binding as peroxyformic acid oxidation imparted an even greater negative charge to the protein. Furthermore, the intermediate inhibitory activity of OFd correlated with its expressed level of C-determinant reactivity, as measured with the C-determinant-specific mAb, 16a. This was seen in Fig. 5 where the level of 16a binding to OFd was intermediate between C-molecule and N-molecule. This lends additional support to the suggestion that Fd11F is specific for the C-determinant of Fd.

That Fd11F should bind to either N- or C-determinant, as opposed to other sites on the molecule, was consistent with the pattern of Fd antigenicity. It is known that all of the B and T cell interactions with Fd reported so far, are focussed at the N- and C-determinants. The significance of the C-determinant specificity of Fd11F, with respect to regulatory mechanisms, cannot be assessed at this time. However, it is of interest to compare Fd11F binding with the determinant specificity of the Ab secreted by the cells used in the suppression assay. As shown in Fig. 9A, all of the anti-Fd Ab produced by the cells obtained from the C-molecule and Fd-immunized mice was directed to N-molecule. The lack of reactivity with C-molecule was not explained by the "hidden" expression of C-determinant due to its binding to solid-phase, as the anti-Fd mAb, 16a (specific for the
C-molecule), was able to bind (Fig. 5). The pattern of Ab activity of the cultured cells was confirmed by the similarity in binding distribution of serum Ab of the mice (Fig. 9B). The results implied that, in vitro, C-determinant-specific Fd11F suppressed the anti-N-determinant Ab response. The designation of C-determinant as the carrier and N-determinant as the hapten of the Fd molecule would be consistent with this observation. Carrier specificity was demonstrated for KLH-TsF (Takemori and Tada 1975) and Ts in the immune response to insulin (Jensen, Pierce, and Kapp 1984). The hapten-carrier model of Fd was also used to explain the effects of anti-idiotypic Ab on the immune response in H-2\(^k\) animals (Weaver et al 1983).

Most of the reported Ag-specific TsF have exhibited Ag-binding activity by their ability to bind to immunoadsorbent columns. Relatively few examples have shown immunochemical properties similar to Fd11F, such as its direct detection using ELISA or the determination of fine specificity of binding using Ag and structural analogs. However, a T cell-derived, Ag-binding molecule described by Cone et al. (1987) does share some features with Fd11F. Using a rabbit antiserum, they detected the molecule in the serum of desensitized mice. It displayed similar immunochemical properties to Fd11F and had a \(M_r\) of about 37k as analyzed by SDS-PAGE, with reducing agent. The fine Ag specificity shown by Fd11F was also seen with two other TsF raised to natural protein Ag: lysozyme (Adorini et al. 1984) and insulin (Jensen and Kapp 1986). These investigators used
immunoadsorbent columns made with structural analogs of the respective Ag to demonstrate specificity of TsF binding.

A more direct relationship between a different Ag system and Fd11F was shown by using an antiserum developed by North et al. (1988). A rabbit antiserum (known as anti-p30) was raised to the 30k $M_r$ protein of Fd11F and shown, using FACS analysis, to bind to not only Fd11 and the A10 hybridoma, but to a proportion of normal murine T cells as well. Anti-p30 reacted with the CD4 and CD8 differentiated T cell subsets in addition to thymocytes that did not express either marker. Related to this work, but in an independent line of study, Askenase and colleagues described T cell-derived, Ag-binding molecules that were found to initiate the CS response (Ptak et al. 1986a,b). In an effort to characterize the origin of the factor-producing cell, they found that T cells from nude mice were capable of producing this Ag-binding factor (Herzog et al. 1989). Subsequently, they derived a cloned cell line from nude mice that reacted specifically with anti-p30 (P. Askenase, unpublished). Whether the antiserum is reactive with a novel type of TCR, possibly expressed on an undifferentiated "primitive" T cell subset, remains to be seen.

**Biologic Properties of Fd11F**

Biologic activity of Fd11F was shown in two ways. Firstly, the addition of Fd11F to Ag-primed spleen cells suppressed the production of anti-Fd Ab. This was in comparison to cultures
containing control material or medium alone. Past experience in this laboratory had shown that it was not technically feasible to couple Fd to sheep red blood cells. Therefore a different spleen cell assay was devised, to replace the conventional plaque-forming cell assay, for the measurement of Fd11F-mediated suppression. Preliminary experiments had also shown that primary cultures of spleen cells produced an insufficient amount of Ab for reliable measurement using ELISA. Therefore, Ag-primed cells would be used for the assay. Furthermore, the spleen cells were pulsed (i.e. cultured briefly with a high dose of Ag) to stimulate the production of Ab in vitro. B10.D2 (H-2d) mice were used as the source of spleen cells in order to test the effect of Fd11F in a syngeneic system. Therefore, as part of the immunization protocol, the B10.D2 animals were made immune to Fd by priming them with C-molecule. The data of Fig. 7 showed that the amount of secreted Ab represented a strong "signal" above background, to measure suppression with.

As the mechanism of action of Fd11F was unknown, several variations in the assay had to be tried before the maximal level of suppression with Fd11F was determined. The results from two protocols were shown in this thesis. The majority of in vitro testing with Fd11F was done with Ag-primed cells that were pulsed with Fd and then continuously co-cultured with Fd11F for the entire incubation period (Fig. 10). This protocol was later modified so that the spleen cells were first incubated with Fd11F and then exposed to Fd, without washing between additions.
Following a 2 h exposure to Fd, the cells were washed and cultured without receiving any further additions.

Suppression of the anti-Fd Ab response by Fdl1F was achieved in a concentration-dependent manner using either protocol. A maximum of 60% suppression was attained using the original assay of Ag pulsing followed by co-culturing with Fdl1F. It should be emphasized that the data of Fig. 10 was compiled using the results from different batches of Fdl1F. The observed curvilinear relationship between percent suppression and Ag-binding activity suggested that both the assay and the Fdl1F preparation technique were reproducible. By revising the protocol, suppression approaching 100% was noted (Fig. 12).

Other investigators have achieved nearly 100% suppression when naive or Ag-primed spleen cells were co-cultured with both soluble Ag and TsF at the same time (Taniguchi, Saito, and Tada 1979; Kapp et al. 1976). For the present work, suppression was observed when the spleen cells were pulsed with factor followed by Ag. The effectiveness of pulsing spleen cells to "arm them" with factor for suppression has been reported by Moser, Kauffman, and Abbas (1985). An alternate explanation, for the effectiveness of Fdl1F pulsing, is provided by the "plus-minus" network theory, proposed by Hoffmann (1980). It was postulated that during the suppressed state, monovalent Ag-binding factors would bind to T cells of complementary specificity and inhibit their mutual stimulation, preventing the induction of an Ab response. When the spleen cells were first exposed to Ag
followed by co-culturing with Fdl1F, the percentage suppression reached a maximum and appeared to plateau (Fig. 10). It is possible that the Ag pulsing period had given the Th a "headstart" towards Ab production before the suppressive effects of Fdl1F began.

Despite the difference in observed levels of suppression, additional experiments using the "original" (Figs. 10 and 11) or "revised" protocol (Table II) supported the idea that the suppressive activity of Fdl1F was Ag-specific. As seen in Fig. 11, Fdl1F did not suppress the Ab response to an unrelated Ag. Table II showed that in reciprocal criss-cross experiments, Fdl1F was suppressive in the Ab culture assay, whereas A10F was not. Conversely, A10F suppressed the CTL response to P815, while Fdl1F did not. It could be argued that although the factors were tested in different cellular assays, the molecules themselves were similar as they were both isolated using the same mAb. In addition, A10F does suppress the syngeneic CTL response to P815 in a concentration-dependent manner and does not alter the magnitude of a mixed lymphocyte reaction with DBA/2 spleen cells on histoincompatible targets (Steele, Chan, Stammers, Singhai, and Levy 1987; Chan et al. 1988). Collectively, the data are consistent with Fdl1F being Ag-specific.

Biological activity of Fdl1F was also shown in vivo. The serum Ab responses of Fd-immune, DBA/2 H-2d mice were suppressed, as compared to control animals, when challenged with Fd and Fdl1F. Due to the wide range in Ab responses of the mice
following their initial immunization, the animals were ranked according to their serum Ab levels and placed into FdllF, A10F, or PBS "treatment" groups prior to receiving factor. In this way, approximately equal numbers of animals having similar pretreatment Ab levels would receive either FdllF, A10F, or PBS. Again, because of the wide range of responses, the data was recorded as individual changes in Ab levels before and after treatment.

It was clear from the data of Fig. 13 that the Ab levels of the FdllF-treated animals had decreased following factor administration and Ag challenge. In contrast, the anti-Fd Ab levels of the PBS or A10F groups had by and large increased. The relatively poor immunogenicity of Fd can probably account for the Ab levels of most of the control group animals that remained close to the "no change" line. Earlier work had shown that a secondary response to Fd develops more slowly as compared to the response to a more complex Ag such KLH. Indeed, the data of Fig. 14, showing the Ab response of the identical animals to KLH, indicated that they were capable of responding readily to this Ag.

It was noted that the timing of factor administration and Ag challenge appeared to be critical for the suppressive effect. In the experiment of Figs. 13 and 14, Ag was given within several hours of FdllF treatment. The inability to give Ag soon after factor resulted in a failure to suppress the response (data not shown). If an immune response is determined by a balance between
help and suppression, then it is possible that the time of delivery of a "helper" or "suppressor" signal can influence the outcome. This is also supported by the difficulties in developing the in vitro assay for the measurement of suppression. In this instance, the order of exposure of the cells to Fd11F and Ag helped to determine the observed level of suppression.

Biochemical Analysis of Fd11F

The biochemical identification of the Ag-binding molecule of Fd11F was made possible because B16G permitted the immunoaffinity enrichment of the active material from Fd11 spent culture SN. The eluted material, as analyzed by reducing SDS-PAGE, showed a limited number of bands that appeared to be unique to the Fd11 culture SN in comparison to control material. A predominant band at 30k $M_r$ was always present in the eluted material (Fig. 15). In addition, a protein at 80k $M_r$ was recovered. Although the band was not unique to Fd11 SN, it was present at greater concentrations in comparison to controls. For this reason, it was initially considered as another possible Fd11F component. There were other, minor components also present in the range of 45 to 50k $M_r$ but because they were present at low concentrations, they were initially not thought to have significance.

In an attempt to isolate Fd11F in its native form, B16G-eluted material was fractionated using high performance gel filtration (FPLC). The Fd11F material was resolved as a single
peak, but unexpectedly, at a $M_r$ greater than 200k. The native $M_r$ of previously reported T cell-derived Ag-binding molecules have not exceeded 145k (Beaman et al. 1984). One explanation was that Fd11F had aggregated under the conditions used for either affinity enrichment or gel filtration. Aggregation of TsF has been previously reported (Webb, Kapp, and Pierce 1983). Besides the intrinsic hydrophobic nature of TsF in aqueous solution, there are other explanations for aggregation. The immunoaffinity column protocol may have caused Fd11F material to denature and aggregate upon recovery under very acidic conditions. This could have been compounded by further concentration of the sample, which was required for high performance gel filtration.

The peak was comprised of fractions that possessed Ag-binding activity. In a separate experiment, suppressive activity was also identified in the vicinity of this high $M_r$ peak. This active material from individual fractions, when analyzed by reducing SDS-PAGE, contained large amounts of both the 80k and 30k $M_r$ proteins described above. One of the peak fractions also contained a pair of bands at about 45k $M_r$. However, the role of these proteins was thought to have little significance for Ag-binding activity because of the low concentrations observed, in comparison to the other protein bands.

Despite the failure to determine the native $M_r$ of Fd11F by gel filtration, the correlation of the 80k and 30k $M_r$ proteins with Ag-binding and suppressive activity was confirmed with these experiments.
The FdllF-enriched material was also fractionated using preparative SDS-PAGE, run under non-reducing conditions. This same technique was used successfully for both A10F (Chan et al. 1988) and GAT-TsF (Sorensen, Pierce, and Webb 1983). As shown in Fig. 20, heavy bands at 80k $M_r$ were recovered from both samples in fraction 4. No Ag-binding activity was associated with these or any fractions in the vicinity; i.e. fractions 1 to 11 (fig. 19) from either Fdll- or DC10-derived material. To determine if the 80k $M_r$ materials were different by some other physical parameter, besides $M_r$, they were analyzed by two-dimensional gel electrophoresis and observed to be identical, within limits of the technique.

However, 30k $M_r$ bands, unique to Fdll material, were detected in fractions 12 to 14. This was coincident with the first Ag-binding activity peak of the Fdll-derived material. A single band was observed in fraction 12, whereas two or three bands were seen in fractions 13 and 14. When preparative SDS-PAGE was repeated, using 10 times the amount of starting sample, the unique 30k $M_r$ material from the fractions was recovered and analyzed by two-dimensional gel electrophoresis. In spite of the numerous corresponding spots shared between the control and FdllF material, there were some unique polypeptides (and one major one in particular) in the FdllF sample at around the 30k $M_r$ position. However, these two-dimensional gel results also demonstrated how heterogeneous the recovered 30k $M_r$ material was (data not shown).

It was therefore shown that an unique 30k $M_r$ protein with
associated Ag-binding activity could be recovered from the Fdl1-derived SN. This moiety itself may have undergone some proteolysis yielding smaller peptide fragments (less than 14k Mr) with Ag-binding activity. An alternative possibility was that the 30k Mr molecule was part of a larger protein that was capable of both Ag-binding and suppressive activity. Since no suppressive activity was ever demonstrated in the recovered 30k Mr material one could conclude that either it was partially denatured or constituted only a part of the TsF molecule. The Ag-binding activity recovered from the bottom of the non-reducing SDS gel (Fig. 19) may be an additional breakdown product. The TsF reported by Fresno, McVay-Boudreau, and Cantor (1982) was susceptible to proteolysis. It degraded into a 24k Mr Ag-binding molecule and a 45k Mr nonspecific suppressor molecule.

Whether Fdl1F is composed of more than one protein component is unknown. It is possible that the 30k Mr protein associates with either the 80k or 45k (fig. 18) Mr polypeptide. Indeed, the "identical" spotting pattern seen with the 80k Mr material, that was analyzed by two-dimensional gel electrophoresis (fig. 21) does not formally rule out that it is a component part of Fdl1F. Conceivably, the elongated spots may actually consist of more than one molecular species and that the "unique" Fdl1F material is hidden. Another possibility is that B16G is specific for a "constant region" of the TsF, and that unique banding at the 80k Mr position between the control and Fdl1 material would not be expected.
Two bands at 45k $M_r$ were identified as unique Fdl1 proteins in fig. 18. An unique 45k $M_r$ component is frequently recovered from the B16G eluates, but it is not always clearly present due to the lower amount of its recovery as compared with the 30k $M_r$ protein (data not shown).

If Fdl1F was actually composed of a 30k and 45k $M_r$ chain, forming a heterodimer, then the two components would be expected to be observed in equimolar quantities, assuming equivalent recovery of the proteins from the imunoaffinity column. There is clear evidence (from two-dimensional gels) that the recovered 30k $M_r$ protein was heavily contaminated with other proteins. Additionally, the non-reducing preparative SDS-PAGE fractionation yielded Ag-binding fractions that contained several polypeptide bands (fig. 20, fractions 13 and 14). Therefore equimolar quantities of both the 30k and 45k $M_r$ components may actually be present in the eluted material, obscured by other contaminating proteins.

The idea of a heterodimeric form of Fdl1F is in keeping with the known structures of reported TsF (eg. Saito and Taniguchi 1984) and the TCR that have been characterized by gene cloning and mAb. At present, it is unknown if Fdl1F is related to any of the heterodimeric TCR molecules: $\alpha/\beta$ or $\delta/\delta$ (Brenner et al. 1986; Saito et al. 1987; Loh et al. 1987). Recent reports have suggested similarities between hybridoma-derived TsF and the $\alpha/\beta$ TCR. Zheng et al. (1988) described an Ag-binding molecule secreted by a Th hybridoma that combined with the I-J$^+$ chain of
an inducer TsF to give suppressive activity. Fairchild, Kubo, and Moorhead (1988) characterized a DNP-specific TsF that reacted with two mAb specific for the products of \( V_\beta 8 \), a family of murine TCR \( \beta \) chain genes. Kuchroo et al. (1988) showed that selection of CD3-expressing Ts\(_3\) hybridomas correlated with the ability to bind Ag and produce TsF. In addition, a mAb specific for the TCR \( \alpha \) chain, immunoprecipitated a protein of the same \( M_r \) (45k) as the \( \alpha \) chain, from the hybridoma cells. (The CD3 molecule, is co-expressed with the \( \alpha/\beta \) TCR on the T cell surface (Clevers et al. 1988)). On the other hand, it is known that Ts (and their soluble factors) differ from the \( \alpha/\beta \) TCR-bearing cell by being able to bind Ag in the absence of MHC (Webb et al. 1989). This suggests that although the Ts receptor may be similar, it is not identical to the \( \alpha/\beta \) TCR.

**Summary**

The TsF-specific mAb, B16G, has been used to isolate Fd11F, a soluble mediator secreted by the Ts hybridoma, Fd11. Although the Ab was originally raised for the characterization of a P815-specific TsF, it has been useful for the study and isolation of TsF in other Ag systems.

B16G was a critical tool in the development of the Fd11 hybridoma. In several respects, this cell line was a good choice as a continuous source for Fd11F. Firstly, Fd11 remained stable for at least four years with respect to the production of Fd11F.
Secondly, the exposure of Fd11 to Fd induces the intracellular influx of Ca$^{2+}$ ions, as measured using a calcium-sensitive fluorescent dye (Steele, Chu, Chan, North, and Levy 1987). This event has been associated with the interaction of ligand with its cell surface receptor (Imboden et al. 1986). Whether Fd11F is a secreted form of a receptor is unknown. However, the calcium flux studies do suggest the presence of a membrane-associated, Ag-specific molecule. Lastly, using FACS analysis, the rabbit antiserum, anti-p30, was shown to bind to the Fd11 hybridoma (North et al. 1988). These data also suggest the presence of a membrane-associated moiety, possibly Fd11F, expressed by Fd11 hybridoma.

The properties of Fd11F were consistent with those shown by other reported TsF. It bound Ag, had suppressive activity both in vitro and in vivo, and has as a part of its molecular composition, a 30k M$_r$ Ag-binding chain.

The main thrust of the present work was the characterization of the Ag-binding molecule of Fd11F. It is acknowledged that several important areas, such as expression of suppressor determinants (eg. I-J) and genetic restrictions for activity were not addressed. However, it is concluded that Fd11F is sufficiently characterized to warrant further investigation. Of interest is the possibility that Fd11F is actually made up of more than one chain. Further biochemical studies are needed to establish the molecular composition of Fd11F and its relationship, if any, to the known TCR products.


Graves, M. C., G. T. Mullenbach, and J. C. Rabinowitz. 1985. Cloning and nucleotide sequence determination of the


of suppressor T cells in nonresponder mice by the terpolymer L-glutamic acid<sub>60</sub>-L-alanine<sub>30</sub>-L-tyrosine<sub>10</sub> (GAT). J. Exp. Med. 140:649-59.


which idiotypic expression does not show linkage to either Igh alleles or the MHC. *Immunology* 51:743-53.


THE COMPOSITIONS OF BUFFERS AND SOLUTIONS

Bicarbonate buffer

1.59 g Na$_2$CO$_3$
2.93 g NaHCO$_3$
0.2 g NaN$_3$
in 1 l of distilled water, adjust to pH 9.6 with HCl;
store at 4°C (Voller, Bidwell, and Bartlett 1976).

Diethanolamine buffer

97 ml diethanolamine
0.1 g MgCl$_2$.6H$_2$O
0.2 g NaN$_3$
in 800 ml of distilled water, adjust to pH 9.8 with HCl;
add distilled water to 1 l; store at 4°C (Voller, Bidwell, and
Bartlett 1976).

Phosphate buffered saline (PBS)

0.137 M NaCl
27.0 mM KCl
71.9 mM Na$_2$HPO$_4$.12H$_2$O
14.7 mM KH$_2$PO$_4$

Phosphate buffered saline containing Tween 20 (PBS-Tween)

PBS
0.05% Tween 20 (v/v) (Voller, Bidwell, and Bartlett 1976).

saturated (NH$_4$)$_2$SO$_4$

900 g (NH$_4$)$_2$SO$_4$ are added to 1 l of distilled water; this is
heated until (NH$_4$)$_2$SO$_4$ is dissolved completely; the hot solution
is then quickly filtered using Whatman No.1 paper; the solution
is cooled at room temperature to allow for crystal formation; at
this time the solution is adjusted to pH 7.4 using NH$_4$OH (Mishell
and Shiigi 1980).

SDS-PAGE Solutions

1) Stock solutions
a) 10% APS

0.05 g APS
500 µl distilled water, prepare daily

b) 30% acrylamide (30% T, 3.3% C, see below)

29 g acrylamide (Bio-Rad)
1 g bis-acrylamide (Bio-Rad)
in 100 ml of distilled water; this solution is filtered (0.45 µm)
stored at 4°C, and protected from light.
Note:
% T = ((g acrylamide + g bis-acrylamide) ÷ total volume) x 100%
% C = (g bis-acrylamide ÷ (g acrylamide + g bis-acrylamide))
x 100%

c) 25% acrylamide (25% T, 2.5% C)

25 g acrylamide
0.65 g bis-acrylamide
in 100 ml of distilled water; this solution is filtered (0.45 µm)
stored at 4°C, and protected from light.

d) stacking (upper) gel buffer (5 x)

0.625 M Tris (Trizma, base, T-1503, Sigma)
0.5% SDS
adjust to pH 6.8 using HCl; store at 4°C.

e) separating (lower) gel buffer (5 x)

1.87 M Tris
0.5% SDS
adjust to pH 8.8 using HCl; store at 4°C.

f) running buffer (10 x)

0.25 M Tris
1.92 M glycine (G 7126, Sigma)
1.0% SDS

2) for 15 ml of a 10% (% T) separating gel solution:

6 ml 25% acrylamide
3 ml lower gel buffer
6 ml HPLC-pure water
mix without introducing air; to polymerize, add:
70 µl 10% APS
7 µl TEMED

3) for 7.5 ml of a 4% (% T) stacking gel solution:
1 ml 30% acrylamide
1.5 ml upper gel buffer
5 ml HPLC-pure water
mix without introducing air; to polymerize, add:
100 µl 10% APS
10 µl TEMED

IEF solutions
1) Stock solutions

a) 10% CHAPS
0.5 g CHAPS in 5 ml distilled water.

b) sample buffer
9.8 M urea
3% CHAPS
2% ampholines
100 mM DTT

c) overlay buffer
8 M urea
3% CHAPS
2% ampholines
100 mM DTT
sample and overlay buffers are aliquoted and stored at -70°C.

d) 30% acrylamide solution (30% T, 2.7% C)
29 g acrylamide
0.8 g bis-acrylamide
in 100 ml of distilled water; this solution is filtered (0.45 µm)
stored at 4°C, and protected from light.

e) anolyte
20 mM NaOH, prepare fresh, degas before use.

f) catholyte

10 mM H$_3$PO$_4$, prepare fresh, degas before use.

2) for 5 ml of IEF gel solution:

2.75 g urea  
0.65 ml 30% acrylamide  
1 ml 10% CHAPS  
0.85 ml HPLC-pure water  
0.36 ml pH 3-10 ampholines

to polymerize, add:
5 µl 10% APS  
3.5 µl TEMED

IEF solutions were adapted from the method of Bravo (1984) with the exception of the CHAPS and 30% acrylamide solution. The use of CHAPS in place of Triton X-100 was originally described by Perdew, Schaup, and Selivonchick (1983). The composition of the 30% acrylamide solution was described in the Bio-Rad technical bulletin 1144.