GENETICS OF THE IMMUNE RESPONSE TO FERREDOXIN.

ASSESSMENT OF CONTROL AT THE DETERMINANT LEVEL.

by

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ABSTRACT

Ferredoxin (Fd), a fifty-five amino acid electron transport protein of the anaerobe Clostridium pasteurianum, has been chosen as the ideal probe for immunoregulation studies. Its critical feature is that it contains two antigenic determinants satisfying the hypothetical minimum requirement for immunogenicity. It was found that both the antibody (as measured by ELISA) and the lympho-proliferative responses to Fd are linked exclusively to the MHC of mice, mapping to K/I-A.

Analysis of the response was undertaken at the determinant level with selective enzyme cleavage products of trypsin and carboxypeptidase A which yield respectively a 52 residue C-determinant peptide (devoid of a functional N-determinant), "C", and a 53 residue N-determinant peptide (devoid of a functional C-determinant peptide), "N". Through the use of these two molecules ("N" and "C") and a doubly digested molecule, "M", the immune response to Fd was dissected.

At first, anti-Fd antibody from high responder, H-2\textsuperscript{k}, mice was shown to be 10-20% "N" specific with the balance of the response "C" directed, while intermediate responder haplotypes, H-2\textsuperscript{b} and H-2\textsuperscript{s}, demonstrate equal specificity for the two determinants. H-2\textsuperscript{d} mice were uniformly non-responsive. Fd immune T-cells demonstrated
lymphoproliferative capacity mirroring the antibody response of B10.BR (H-2^k) mice: "C" induced proliferation comparable to that with the native molecule, "N" inducing a much lower response, one matched by the "M" peptide.

Next, the "N", "C" and "M" molecules were assessed for their immunogenicity in B10.BR, C57BL/10 (H-2^b) and B10.D2 (H-2^d) mice. "N" was found to induce limited, if any, antibody production whereas it primes for a very good proliferative response (B10.BR only). "M" induced no antibody response in any strain, and minimal proliferation in B10.BR. "C" induced at least two-fold higher antibody in B10.BR and C57BL/10 as compared to native Fd, and converted the B10.D2 non-responders into responders. "C" induced a weak proliferative response in B10.BR.

The data suggest that two determinants exist at the B-cell level, while three determinants account for the T-cell response.
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<td>anti-B0</td>
<td>anti-brain associated θ-antigen</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<td>A-25</td>
<td>DEAE-Sephadex (Pharmacia)</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>&quot;C&quot;</td>
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<td>C'</td>
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<td>two Fd C-determinants linked by 12 glycines</td>
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<td>DNP</td>
<td>dinitrophenyl</td>
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<td>DPDM</td>
<td>N,N'-p-phenylenedimaleimide</td>
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DTH - delayed-type hypersensitivity
FCS - foetal calf serum
Fd - ferredoxin
FITC - fluoroisothiocyanate
GA - glutamine, alanine random copolymer
GAT - glutamine alanine tyrosine random copolymer
GA-T - GA with COOH-terminal tyrosine
GA-(T)² - GA with tyrosine sidechain
GEL - gallinaceous egg-white lysozyme
GL - glutamine lysine random copolymer
GLA - glutamine lysine alanine random copolymer
GLleu - glutamine lysine leucine random copolymer
GLφ - glutamine lysine phenylalanine random copolymer
GPA - guinea-pig albumin
GT - glutamine, tyrosine random copolymer
GZ - β-galactosidase
G-50 - Sephadex gel (Pharmacia)
Hb - haemoglobin
HEL - hen egg-white lysozyme
(H,G)-A--L - poly-L-(histidine, glutamine)-poly D, L-Alanine-poly-L-lysine
HR - high responder
HSA - human serum albumin
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<td>Ia</td>
<td>Ir gene product, antigen</td>
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<td>Id</td>
<td>idiotype</td>
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<tr>
<td>IFA</td>
<td>incomplete Freund's adjuvant</td>
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<td>IgCH</td>
<td>heavy chain allotype</td>
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<tr>
<td>Ir</td>
<td>immune response gene</td>
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<td>Ir</td>
<td>intermediate responder</td>
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<td>LDH&lt;sub&gt;B&lt;/sub&gt;</td>
<td>lactate dehydrogenase B</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LR</td>
<td>low responder</td>
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<td>Lyt-1</td>
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<td>&quot;M&quot;</td>
<td>CPA and TR Fd digest product, middle peptide with no function N- or C-determinants</td>
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<td>methylated oxidized Fd</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>macrophage inhibitory factor</td>
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<td>MSA</td>
<td>mouse serum albumin</td>
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<td>M.W.</td>
<td>molecular weight</td>
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<td>CPA digested Fd with only a functional N-determinant</td>
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<td>Nase</td>
<td>staphyloccocal nuclease</td>
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N+C-S-BSA - Fd N- and C-determinants linked to BSA
NEM-Fd - N-ethyl maleimide alkylated Fd
NR - non-responder
N-S-BSA - Fd N-determinant linked to BSA
N-5-C - N- and C-determinant bridged by 5 glycine residues
N-8-N - two N-determinants bridged by 8 glycine residues
N-10-C - N- and C-determinant bridged by 10 glycine residues
O-Fd - oxidized Fd
PETLES - peritoneal exudate T lymphocyte enriched cells
PFC - plaque forming cells
(Phe,G)-A—L - poly-L-(phenylalanine, Glutamine)-poly-D,L,alanine--poly-L-lysine
PLL - poly-L lysine
Poly 18 - poly-glu-tyr-lys-(glu-tyr-ala)5
PPD - purified protein derivative
RBC - red blood cell
REL - ring-necked pheasant egg-white lysozyme
RIA - radio-immunoassay
S.I. - stimulation index
SRBC - sheep red blood cell
(T-A-G-Gly)n - ordered polymer (Tyr-Ala-Glu-Gly)n
TCA-Fd - trichloroacetic acid reduced Fd
(T-G-A0Gly)n - ordered polymer (Tyr-Ala-Glu-Gly)n
(T,G)-P--L - poly-L-(Tyr,Glu)-poly-D,L,proline--poly L-Lys
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<td>TNP</td>
<td>trinitrophenyl</td>
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<tr>
<td>TR</td>
<td>trypsin</td>
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<td>G-90</td>
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In memoriam

GENERAL TADEUSZ KASPRZYCKI
Chapter 1. Introduction

I. Historical Review: Ferredoxin

Ferredoxin (Fd), an electron transport protein of the anaerobe, *Clostridium pasteurianum*, has been the subject of intensive study in Dr. J.G. Levy's laboratory since the mid-1960's. It was chosen as an immunological probe for its small size and molecular simplicity. The 55 amino acid molecule contains eight more or less evenly spaced cysteiny l residues that are not involved in disulfide linkage in the native molecule and have been found not to be critically involved in antibody binding (1); from crystallographic studies (2) it was determined that in its native form, Fd assumes a hairpin-like configuration in which five iron molecules are chelated through the sulfhydryl groups of cysteine.

As a result of these observations, the areas not containing the cysteine residues were synthesized: the carboxy-terminal pentapeptide \( \text{NH}_2\text{-ala-pro-val-gln-glu-COOH} \) (3), the amino-terminal pentapeptide \( \text{NH}_2\text{-ala-tyr-lys-ile-ala-asp-ser-COOH} \), and sequences in the mid-section of the molecule within the cysteine-rich region (4). These molecules were tested for their ability to inhibit complement fixation between oxidized Fd (OFd) and specific rabbit antisera (4,5); it was found that the \( \text{NH}_2\) - and COOH-peptides bound different populations of antibodies. Furthermore, equilibrium dialysis experiments demonstrated that these two peptides account for essentially all of the antibody synthesized by rabbits to OFd (4). Extensive studies (6,7,8) with an assortment of synthetic peptides representing the N- and C-determinants linked onto
carrier molecules or bridged by a glycine sequence peptide (N-S-BSA, C-S-BSA, N+C-S-BSA, N-5-C, N-10-C, N-8-N, C-mal-10-C, C-12-C) analyzed the specificity of these molecules in a number of assays describing immunity to OFd (immediate and delayed-type hypersensitivity, inhibition of macrophage migration [MIF] and lymphocyte proliferation). The analysis and compilation of these data has been reviewed elsewhere (9). The suggestion was made that the N- and C-determinants are recognized both at the T- and B-cell levels but that the N-determinant appears to be the stronger controlling element of the immune response, presumably via the generation of help. In a carefully carried out study (10) (which had unfortunately been performed in BALB/c mice, which have subsequently turned out to be non-responders to Fd), non-immune intact and anti-brain-associated theta antigen (anti-BA0) treated spleen cells were scored by autoradiography for the binding of $^{125}$I-labelled N-10-C and N-8-N (synthetic peptides containing either the N- and C-determinants separated by a 10 amino acid bridge or two N-determinants bridged by 8 amino acids), the results indicating that the majority of cells binding these molecules were bone marrow derived. Mice were then immunized with N-10-C or N-8-N and the autoradiography assays demonstrated that the N-determinant was equally bound by T- and B-cells, while the C-determinant binding was correlated to numbers of B-cells. It was determined that both the thymus and bone marrow participated in the C-mal-10-C response indicating that the C molecule also stimulates a T-cell population. It was subsequently shown (11), using antigen suicide technology, that separate T-proliferative populations were susceptible to this treatment.
and that the proliferative response was partially restored by mixing the two treated populations. Further work using both ferredoxin subjected to a number of modifications (12), and amino terminal peptides of varying lengths (13), suggested, albeit inconclusively, that T-cell and B-cell repertoires, with respect to recognizing and binding antigenic determinants, may be different.

It was in the interest of placing the above, and subsequent research with ferredoxin in perspective with studies in other systems, that a review of antigenic probes used in immunoregulatory and genetic studies was undertaken. Only the facts critical to aspects of the present research will be reviewed. It must also be kept in mind that as the majority of the studies under discussion have been reported on prior to the presentation of the "new version" of the H-2 complex by Klein and co-workers (14) the following review will still employ the "traditional" nomenclature of I-A, I-B, I-J, I-E and I-C. However, in the discussion, the data will be interpreted in terms of the new version.

II. Historical Review: Other Antigens

Since the 1930's, scientists have been developing an interest in phenomena whose elucidation forms the foundation of the field of immunology: a) Landsteiner's description of antibody specificity (15) b) Benacerraf's group's linking the lack of antibody production to a specific antigen in certain outbred animals to genetic inheritance (16) and c) the subsequent discovery that the immune response (Ir) genes are
### Table I - Immune responses to protein antigens

<table>
<thead>
<tr>
<th>Group/number</th>
<th>Antigen</th>
<th>Molecular Weight</th>
<th>Number of determinants</th>
<th>Responder/Non responder</th>
<th>Ir loci References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>PLL</td>
<td>one</td>
<td></td>
<td>R = g.p.2</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR = g.p.13</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>PLA</td>
<td></td>
<td></td>
<td>R = g.p.2</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR = g.p.13</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>GL</td>
<td>~60,000</td>
<td></td>
<td>R = g.p.2</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR = g.p.13</td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>GA</td>
<td>~45,000</td>
<td></td>
<td>R = g.p.2; a, b, d, j</td>
<td>22, 25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>k, u, v</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR = g.p.13; u, p, g.</td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>GT</td>
<td></td>
<td></td>
<td>R = g.p.13</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR = g.p.2</td>
<td></td>
</tr>
<tr>
<td>A6</td>
<td>GLA^5</td>
<td>25,000 - 75,000</td>
<td></td>
<td>R = d, f, s</td>
<td>I-B 23, 24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IR = a, k, q</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LR = b</td>
<td></td>
</tr>
<tr>
<td>A7</td>
<td>GLA^30</td>
<td>25,000 - 75,000</td>
<td></td>
<td>R = a, b, d, f, j</td>
<td>25</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>k, u, v</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR = u, p, q</td>
<td></td>
</tr>
<tr>
<td>A8</td>
<td>GLT</td>
<td></td>
<td></td>
<td>R = d, j, a, q, r</td>
<td>I-C 29, 55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR =</td>
<td></td>
</tr>
<tr>
<td>A9</td>
<td>GLO</td>
<td></td>
<td></td>
<td>R = d, j, p, q, v, j</td>
<td>I-A/B 44, 285</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>a, b, k, s, f, v, h4</td>
<td>and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IR = a, b, k, s, f, v</td>
<td>I-C (*)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(c)</td>
</tr>
<tr>
<td>A10</td>
<td>GAT^10</td>
<td>~25,000</td>
<td></td>
<td>R = a, b, d, k</td>
<td>I-A/B 27, 28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR = f, j, p, q, r,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>a, w</td>
<td>(*) 29, 30</td>
</tr>
<tr>
<td>A11</td>
<td>(T-A-G-</td>
<td>~33,000</td>
<td>presumably one</td>
<td>R = g.p.2; b, f, r</td>
<td>I-A 57, 58</td>
</tr>
<tr>
<td>Gly)n</td>
<td></td>
<td></td>
<td></td>
<td>NR = g.p.13</td>
<td>(*) 59</td>
</tr>
<tr>
<td>A12</td>
<td>(T-G-A-</td>
<td>~53,000</td>
<td>presumably one</td>
<td>R = g.p.13; b</td>
<td>I-A 58, 59</td>
</tr>
<tr>
<td>Gly)n</td>
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<td></td>
<td></td>
<td>NR = g.p.2</td>
<td>(*)</td>
</tr>
<tr>
<td>Code</td>
<td>Protein</td>
<td>MW</td>
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<td>------</td>
<td>---------</td>
<td>----</td>
<td>-----</td>
<td>------------------------</td>
<td></td>
</tr>
<tr>
<td>A13</td>
<td>Poly 18</td>
<td>11,000</td>
<td>$R = d$&lt;br&gt;$NR = k,b;g.p.13$</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>A14</td>
<td>(T,G)-A</td>
<td>60,000</td>
<td>at least one &lt;br&gt;$R = b,s$</td>
<td>I-A (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- L</td>
<td>180,000</td>
<td>$NR$</td>
<td>I-A and I-E/C</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>Albumin</td>
<td>66,210</td>
<td>5-16 &lt;br&gt;$R = g.p.2;k,a,d$</td>
<td>I-A and I-E/C</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$LR = g.p.13;b$</td>
<td>22,99</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>Collagen, type I (gelatin)</td>
<td>50,000</td>
<td>2</td>
<td>(at least) 105,106</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$R = b,f,s$</td>
<td>I-A (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$LR = a,d,k,m,$&lt;br&gt;$p,q,r,u$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>Cytochrome $c$ (pigeon)</td>
<td>12,350</td>
<td>3</td>
<td>I-A and I-E/C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$HR = k,a$</td>
<td>118,119</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$LR = b,d,p,a,s,$&lt;br&gt;$u,f,j,a,r$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B4</td>
<td>Fibrinopeptide</td>
<td>2,100</td>
<td>1</td>
<td>left of 127,130</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$HR = g.p.2;k$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$LR = g.p.13;b,d,q$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B5</td>
<td>$B$ Galactosidase</td>
<td>465,000</td>
<td>many &lt;br&gt;(CB-2 major)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>Glucagon</td>
<td>4350</td>
<td>2</td>
<td>I-A and D-end(?)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$R = g.p.2,13$</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>B7</td>
<td>Haemoglobin</td>
<td>64,500</td>
<td>HR = b,d</td>
<td>I-A and I-A</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$LR = j,k,a,r,s$</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$HR = b,d$</td>
<td>I-A (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$LR = k,s,j,f,$&lt;br&gt;$r,u,v$</td>
<td>137,139</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hb $\alpha$ subunit</td>
<td></td>
<td>$HR = b,d$</td>
<td>I-A (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$LR = k,s,j,f,$&lt;br&gt;$r,u,v$</td>
<td>137,139</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hb $\beta$ subunit</td>
<td></td>
<td>$HR = d,f,s$</td>
<td>I-A (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$LR = k,p,r,v$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$LR = b,j,q,u$</td>
<td>137,139</td>
<td></td>
</tr>
<tr>
<td>B8</td>
<td>Hepatitis B antigen</td>
<td>25,000</td>
<td>1 major &lt;br&gt;serological</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B9</td>
<td>Insulin (pork)</td>
<td>5,750</td>
<td>2</td>
<td>I-A and another (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$HR = g.p.2,13;d$</td>
<td>142,143</td>
<td></td>
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<td></td>
<td></td>
<td>$LR = b,v,a,k,$&lt;br&gt;$w,r,s,q$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insulin (beef)</td>
<td>5,750</td>
<td>2</td>
<td>I-A and another (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$HR = g.p.2A_{13B}$&lt;br&gt;$d^B,b^A,v$</td>
<td>142,143</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$LR = a,k,u,v,r,s,q$</td>
<td>146,148</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proinsulin (pork)</td>
<td>9,000</td>
<td>3</td>
<td>I-A and another (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$HR = d,b,k,s$</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$LR = q$</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>B10</td>
<td>Lactate dehydrogenase B</td>
<td>140,000</td>
<td>many</td>
<td>HR = d,q,b</td>
<td>I-Bor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NR = k,a</td>
<td>I-A &amp; I-E</td>
</tr>
<tr>
<td>B11</td>
<td>Lysozyme</td>
<td>14,300</td>
<td>3</td>
<td>HR = f,k,p,d</td>
<td>I-A and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LR = b,s,r</td>
<td>I-C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(*)</td>
</tr>
<tr>
<td>B12</td>
<td>Myoglobin (sperm whale)</td>
<td>17,814</td>
<td>5</td>
<td>HR = d,f,s</td>
<td>I-A and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LR = b,k,p,q,r</td>
<td>I-E/C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(*)</td>
</tr>
<tr>
<td>B13</td>
<td>Staphylococcal nuclease</td>
<td>16,800</td>
<td>2</td>
<td>HR = a,f,d,k,r,s</td>
<td>I-Bk,a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LR = b</td>
<td>I-Ad</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(*)</td>
</tr>
</tbody>
</table>

(a) R = responder strains, NR = non-responder, HR = high responder, LR = low responder, IR = intermediate responder, g.p. = guinea pig strains 2 or 13, all others designate mouse strain haplotypes i.e. a,b,d,f,k,p,r,s...

(b) Ir gene designations as used in the manuscript quoted.

(c) (*) designates that non-H-2 gene involvement was found in the control of the response.
linked to the MHC (17,18). In the last decade, the genetic and molecular bases for cellular interactions in humoral and cellular immunity have been avidly studied with numerous antigenic probes listed in Table 1. It was found that proteins of known chemical structure are the most amenable probes for understanding the nature of antigenic determinants as seen by T- and B-cells, the genetic regulation of the immune response at the determinant level, as well as networks of immune responsiveness utilizing both hapten-carrier and idiotypic interactions. A brief review of the use of synthetic antigens (A) will be followed by an introduction to the major protein antigens (B) used to date in the study of immunoregulation.

A. Synthetic molecules

Early studies on the genetic control of the immune response in the mouse (and guinea pig) have employed a variety of synthetic random copolymers of L-α-amino acids (#A1 - A10, Table I), synthetic polymers of defined geometry (#A11 - A13, Table I), as well as branched multichain copolymers (#A14, Table I).

1. Linear random copolymers

The first linear synthetic antigen to be studied extensively was poly-L-lysine (PLL) and the response to it was found to be under Ir gene control: strain 2 but not strain 13 guinea pigs responded to \( \alpha_{1\text{DNP-lys}} \) (19); this response was shown to be of exquisite antigenic specificity related to the number of lysines and the location of the haptenic moiety; the study also indicated that T- and B-cells have comparable repertoires of specificity (20). It was recently shown that
the gene defect in the non-responding strain is limited to T-cells and not macrophages and B-lymphocytes (21). The gene controlling the PLL response was also found to control the PLA and GL responses in guinea pigs: present in strain 2 and absent in strain 13 (19). Later, strain 2 was also found to be responsive to GA, low doses of bovine serum albumin (BSA) and horse serum albumin (HSA), whereas strain 13 responded to GT (22).

In murine systems, with a much larger number of strains available including congenic and recombinant mice, the true complexity of the response to synthetic copolymers was demonstrated in numerous publications concerning the mapping of the response genes to variants of these copolymers. For example, the terpolymer GLA with a low level of alanine substitution, GLA$^5$ and GLA$^{10}$, was found to be immunogenic in H-2$^{d,f,s}$ strains, showed intermediate immunogenicity in H-2$^{a,k,q}$ and was weakly or non-immunogenic in H-2$^b$ (23); this response was subsequently mapped to the 1-B subregion (24). However, when the level of alanine substitution was increased as in GLA$^{30}$, the response to it became similar to that to the GA copolymer: GLA$^{30}$ and GA were found to be immunogenic in H-2$^{a,b,d,f,j,k,u,v}$ and non-immunogenic in H-2$^{n,p,q}$ (25). However, later studies by the same team demonstrated using GLA$^{20}$, GLA$^{40}$ and GLA$^{60}$ that a highly complex immune response existed to GLA that was antigen dose dependent and multideterminant specific in nature: GLA with low substitution behaved like the GL molecule however, with higher substitution (GLA$^{40}$, GLA$^{60}$), certain haplotypes responded to GL and GA determinants while others responded to unique GLA determinants (26). It is felt that although these molecules were useful in the early
definition of Ir genes, further studies ended up with extremely confusing data which did not serve to clarify the genetics of the immune response.

Copolymers which have since been found to be more useful are GLT, GLleu, GLΦ and GAT, which to date has turned out to be the most immunogenic and widely studied synthetic antigen. The response to GAT was found to be linked to the α, β, δ and κ haplotypes while f, j, p, g, r, s and w strains were non-responders (27,28). This distinction between responders and non-responders was not altered for GAT as opposed to the responses seen with GLA (26) when different doses of antigen were used. In order to pinpoint the antigenic determinants on GAT, cross-reactivity of anti-GAT antisera with copolymers of GT and GAT(T) was studied (28). It was found that all responders reacted to the GA part of the molecule while there was variable genetically controlled cross-reactivity to GT. Subsequent research mapped the response to GAT to the I-A/B subregion (29), although non-H-2 linked control was also implicated, as responder strains on the A background demonstrated at least 10-fold higher antibody binding capacity than did strains on the B10 background (30). Antibodies that were produced in both A and B10 background strains had the same patterns of specificity for GA and GT; this magnitude effect was found to be dominant and linked to the heavy chain allotype loci (IgCH). In subsequent studies where isoelectric focusing was performed on anti-GAT antibodies, it was found that at least in BALB/c mice the response is of restricted heterogeneity (31).

Originally it was proposed that unresponsiveness to GAT in DBA/1 mice was a result of tolerance induction in T-cells (32). Shortly
thereafter, this non-responsiveness was attributed to the action of suppressor T-cells (33). As an extension of the studies with GAT, the response to GT was analyzed and all strains tested were found to be non-responsive to it, yet like GAT, immunization with GT-MBSA induced a response (34). Work carried out by other researchers may in part explain this widespread non-responsiveness (suppression) induced by immunization with GT: H-2^S mice, are responsive to GA but non-responsive to GAT, this may implicate tyrosine as a residue involved in inducing suppression (35). With the use of (GA)-T, evidence was presented that tyrosine at the COOH-terminal actually induces a suppressive function in (G,A) responder mice. The suppression to GT and GAT was subsequently mapped to a region between I-B and I-C (34) and the cell responsible for this suppression was found to be cyclophosphamide sensitive (36).

Responder x non-responder (R x NR)F^1 mice were shown to possess phenotypic helper as well as suppressor cells whose activity depended, respectively, on the presence of macrophages from either the responder or non-responder parent (37). In vitro presence of macrophages was demonstrated to be crucial to the response of high responder cells to GAT; delaying their addition to cultures was shown to induce suppression (38).

Further in vitro studies successfully led to the production of suppressor factors (39): it was found that under stringent conditions these factors could be produced both in responding and non-responding strains, they could induce suppression in all strains in a genetically unrestricted fashion. Other researchers indicated that the factor had to
be present before antigen in order to induce a second set suppressor cell (Ts2) in responder strains (40).

Other in vitro studies demonstrated that GAT–specific suppressor and helper functions are a result of the manipulation of culture conditions and not actual Ir gene controlled phenomena, as under a set of identical conditions, GAT–specific T-helper cells could be obtained in cell cultures from both high and non-responding strains (41). When the T-proliferative assay was employed, strict dependence on macrophages was demonstrated, yet presence or absence of proliferation in non-responder or (NR x R)F1 lymph node cells was dependent on the priming protocol and not on presentation of antigen in culture (42).

The response to GLΦ and GLT (29) and GLleu (43) were found to be multigenic and subsequently found to map to the I-A/B and I-C subregions (44) for the GLΦ response, as H-2^a,b,k,s mice were found to be non-responders, but selected F1 strains between two non-responding strains, (H-2^a x H-2^b)F1 and (H-2^k x H-2^s)F1 were found to produce anti-GLΦ antibody. The complementing genes for the GLΦ response were found, in double adoptive transfer experiments, to be present together on both T- and B-cells (45) with a genetic preference for the cis position (46). Recombinant strains possessing the two appropriate Ir genes for responsiveness were found to produce a higher response than (NR x NR)F1 responder mice; this effect was found to be the result of gene dosage and not cis–trans phenomena (47). GLΦ non-responders were found to make neither IgM nor IgG (48).
More recent work showed that the proliferative response to these peptides followed the same multigenic control pattern (49); it was also found that the antigen presenting cell (50,51) and not the proliferating T-cell (52) needed to carry both complementing genes. This proliferation was inhibited by both anti-I-A and anti-I-E/C antisera (53) which eventually indicated that the restriction element was composed of the β chain encoded in I-\(^{b}\) and the α chain encoded by I-E\(^{k,d,p,r}\) (54).

All these results indicate that both GLΦ and GAT are comparatively good antigens for genetic studies as compared to the GLA copolymers described above and to the closely related copolymer GLT. At first the response to GLT was thought to be similar to that observed with GLΦ in that the control seemed to be multigenic and responsiveness was linked to H-2\(^{d,j,a,q}\) (55). However, more in-depth analysis of the GLT response revealed that GLT is an extremely unstable and variable copolymer making repeatable experiments very difficult (56). In addition, it seems that each responder haplotype recognizes different determinants on the polymer, reminiscent of the situation with GLA (26).

2. Sequential polymers of defined geometry.

The next set of antigens found to be under Ir gene control includes (T-A-G-Gly)\(_n\) which is immunogenic in guinea pigs, strain 2 but not strain 13 responding (57), and mice, H-2\(^{b,f,r}\) mice responding, one gene mapping to I-A and a second locus is found to be non-H-2 associated and responsible for the magnitude of the response (58,59). Anti-(T-A-G-Gly)\(_n\) antiserum, which is entirely IgG, was found not to cross-react with (T-G-A-Gly)\(_n\) and the random copolymers, GA, GT, GAT, nor
the branched copolymer (T,G)-A—L, suggesting discreet determinants and
demonstrating the improvement obtained in the use of sequential as opposed
to random copolymers of α-amino acids for studies on immune regulation.
The response to the non-cross-reactive polymer of similar amino acid
composition but different ordering, (T-G-A-Gly)n, is under even stricter
I$r$ gene control, only $H-2^b$ mice responding; both the humoral and
T-proliferative responses were found to map to I-A. When secondary immune
responses in these $H-2^b$ mice were studied, non-$H$-$2$ effects were quite
apparent in the humoral but not in the proliferative responses. A gene
dose effect was seen in (R x NR)$_n$'s, indicating co-dominance of the
genes (60). Unfortunately, these two non-random, sequential molecules,
(T-G-A-Gly)n and (T-A-G-Gly)n, assume rigid α-helical configurations
which seem to grant them peculiar B-cell mitogenic properties which make
some of the interpretations of the data a little uncertain (59).

Another synthetic polymer of defined α-helical geometry that
is under Ir gene control is Poly 18; it was used in the study of
hapten-carrier responses (61). The haptens TNP or BOC-gly-ARA-tyr were
joined to the Poly 18 backbone at precise intervals thus ensuring limited
numbers of determinants. Responsiveness to TNP-Poly 18 on an immunogenic
carrier (MBSA, insulin, Ficoll, fowl γ-globulin and BOC-gly-ARA-tyr were
tested), by Poly-18 non-responders, $H-2^k,b$, could not be achieved, but
BOC-gly-ARA-tyr-Poly 18 did convert non-responder strain 13 guinea pigs
into responders, suggesting a complex recognition mechanism or very
specific "holes" in the receptor repertoire, although the authors argue
against the possibility of a B-cell defect.
3. **Branched multi-chain co-polymers.**

Another set of synthetic antigens that have been used extensively in the study of immune regulation are branched multichain co-polymers of α-amino acids originally synthesized in the early 1960's (62), the best known being (T,G)-A--L and its close molecular relatives (Phe, G)-A--L and (H,G)-A--L. A controversy existed in the mid 1970's as to the number of loci responsible for the control of the immune response to (T,G)-A--L, although early mapping of the Ir-1 locus suggested a unique gene for the response to (T,G)-A--L (63). Subsequent studies on *in vitro* and *in vivo* antibody production (64) as well as T-cell proliferation (65) described regulation by two complementing genes. This work was repudiated by more extensive screening of the antibody response (66) and more recently, with definite mapping of the response to only I-A (67). The proponents of the two loci control acknowledged subsequently that their findings were faulty (68). In another study a non-H-2 gene was described which controls the IgM response to (T,G)-A--L (a molecule which is capable as acting either as a T-dependent or T-independent antigen), while the IgG, T-dependent response is under Ir gene control (69). Adult thymectomy blocked the IgG but not IgM response (70).

In order to describe the antigenic determinants present in (T,G)-A--L, a molecule that is the result of random polymerization of the (T,G) side chain, ordered tetrapeptides of Tyr and Glu were used (71). Tyr-Tyr-Glu-Glu was found to be an important determinant of (T,G)-A--L as well as the major determinant in the anti-pro response of (T,G)-Pro--L (not under H-2 linked Ir gene control (72)). Subsequently, it was shown...
that changing the order in the tetrapeptide of two amino acids changed the genetic control pattern as compared to the identical genetics observed in response to the random sidechain of \((T,G)-A--L\) and the ordered tetrapeptide of \((T-T-G-G)-A--L\) (73) the tyrosine residue was found, in a separate study, to play an important role in the recognition mechanism of the \((T,G)-A--L\) molecule by the immune system (74).

Extensive studies on the interaction of Ir gene products at the macrophage, T- and B-cell levels were undertaken. One interesting analysis involved two high responder strains, \(H-2^b\) and \(H-2^s\), which were tested for their ability to present antigen to T-helper cells (75). It was cleverly demonstrated that these two high responders do not cross-react, i.e. T-cells from \(H-2^b\) high responders only help the \((TNP)-(T,G)-A--L\) PFC response of \(H-2^b\) B-cells and macrophages.

The \(H-2\) restriction of macrophages was a phenomenon observed quite early in the studies with \((T,G)-A--L\) and a number of closely related antigens (76,77): only high responder macrophages could present antigen to high responder T- and B-cells, whereas low responder antigen presenting cells (APC) could not. High responder macrophages could not induce low responder B-cells to produce antibody, therefore the defect was localized at least at the low responder B-cell and restriction was observed at the APC level. Studies, using helper factors derived from T-cells, addressed the question as to what cell type expresses the Ir gene defect in the recognition of the factor (78). The results indicated a symmetry between T-cell-macrophage and B-cell-macrophage interactions; \((T,G)-A--L\) specific helper factor was found to act on macrophages first and then on B-cells in
a genetically restricted fashion providing a responder or $F_1$ macrophage source was used.

Studies in a number of systems have demonstrated that APC's present antigen in association with Ir gene products. Experiments with (T,G)-A--L and related molecules have demonstrated that T-cells have high affinity for antigen-self Ia complexes, and very little, if any, affinity for antigen alone (79).

With respect to T-cell and B-cell repertoires, in the analysis of responses to two polymers related to (T,G)-A--L, (phe,G)-A--L and (H,G)-A--L, it was found that although antibodies to the three molecules cross-reacted with each other in a similar fashion that antibodies to GLΦ, GLT and GLPro did (22), the T-proliferative responses were specific for the immunizing antigen (80). This differential antigen recognition by T- and B-cells was duplicated using a tolerization protocol where specific tolerance could be established to (T,G)-A--L, but immunization with (Phe,G)-A--L could still induce an antibody response that was limited to (Phe,G)-A--L specific antibody. The antibody cross-reactive to (T,G)-A--L normally produced by (Phe,G)-A--L immunization was not produced by the (T,G)-A--L tolerant mice (81).

A conclusion that may be contested by the researchers utilizing the above-mentioned synthetic antigens is that although the use of these molecules opened up the field of immunogenetics, these molecules are inappropriate in subsequent analyses of the determinant-specific and idiotypic responses to an antigen. This point is supported by recent evidence using monoclonal antibodies in that, not only is it difficult
with these molecules to isolate determinant specific responses, but that a majority of these molecules with similar amino acid content but varying geometric arrangements produce a large amount of cross-reactivity with one another. In this study, monoclonal antibodies were produced to GAT and (T,G)-A--L. It was found that some of the (T,G)-A--L specific antibodies bound the polymers GAT and GT while only some bound (T,G)-A--L exclusively and none bound GA; those monoclonals reacting with GAT also cross-reacted with the three known idiotypes of GAT (82). It is therefore not surprising to find that these researchers experienced difficulties in mapping the genes responsible for the immune response to a number of these molecules. Not wanting to belittle the advances in the understanding of such phenomena as suppression in systems utilizing synthetic antigens such as GAT (83), it can be seen that unless proteins with limited and clearly defined antigenic moieties are used, definitive studies expanding the work pioneered with the use of these synthetic molecules cannot be fulfilled. It is therefore not surprising that much of the more recent immunogenetic studies are concerned with the use of naturally occurring proteins of defined sequence and geometry.

B. Natural Proteins

Table I, numbers B1 - B13, comprises a list of naturally occurring proteins which have been used in immunogenetic and cellular studies. The following section deals with each individually and summarizes the pertinent facts obtained to date concerning the immunoregulation of the response to these molecules.
1. Albumin. Based on proposals as a result of elegant work with staphylococcal nuclease (84,85,86) and sperm whale myoglobin (84,87), it would be expected that Ir gene control could only be established for molecules exhibiting a small number of antigenic determinants. Due to its large size (66,000 m.w.), it could be expected that assignment of immune response genes to bovine serum albumin would be difficult. However, the albumins have been a popular antigen since the early descriptive experiments concerning immune response genes: it was found that with limiting doses of immunogen ($\leq 1$ µg), strain 2 guinea pigs respond to BSA, DNP-BSA, HSA and not DNP-GPA, while strain 13 guinea pigs exhibit the reverse pattern of responsiveness (22).

More recent studies in several laboratories using this complex, globular, three domained molecule, have succeeded in analysing the response with a surprising amount of detail. In one study, the 3 domains were isolated and used to purify domain specific antibodies which showed the domains to be antigenically distinct from one another and to contain each, a minimum of two antigenic determinants, suggesting a minimum of six different non-repeating determinants in the intact molecule (88). These results were seemingly contradicted by another study which found that BSA contains repeating identical or very similar antigenic determinants (89).

The above dichotomy seems to be resolved when one correlates the above with subsequent data from several laboratories: although in most cases, 5-6 B-cell determinants can be isolated, a large amount of cross-reactivity exists between them, indicating that common sequences or configurations may be dispersed throughout the molecule, yet individual
determinants are present in the different domains. In one analysis attempting to reconcile the variations in data, rabbit anti-BSA sera had been produced from individual animals in a span of over one year: the cross-reactivity to albumins from other sources was found to increase dramatically, yet the affinity of the antibody increased and the heterogeneity decreased (90). This was felt to answer the lack of agreement obtained in the different laboratories.

Other problems with the correlation of data stemmed from the fact that the response against native BSA was seriously affected by any chemical changes induced on BSA, as the B-cell but not T-cell response was found to be highly dependent on charge, conformation and degree of aggregation of the molecule (91). In a later study, the antibodies to native and denatured BSA demonstrated absolutely no cross-reactivity, whereas absolute cross-reactivity at the T-cell level was elegantly demonstrated with BUdR and light experiments where 90% of the cross-reactive response was inhibited by this treatment indicating that T-cells recognize sequential segments of BSA (92).

A fortunate aspect was discovered by Atassi and co-workers that makes comparisons between different laboratories easier: through the use of five synthetic sites of BSA (93) and HSA (94), it was demonstrated that mouse anti-BSA antibodies recognize the same sequences bound by rabbit anti-BSA antibodies (95). It could then be predicted that recognition of the sites is independent of the responding species, but is a property of the conformational and structural uniqueness of the five antigenic sites.
A further related aspect was uncovered in another study: autosensitization was assessed in a TNP-MSA model system (96). It was found that an anti-TNP PFC response could be generated in b and d animals, whereas the a and k alleles yielded low responsiveness indicating that autoresponsive clones are present and that their expression is genetically controlled at the H-2.

Further analysis by Atassi and co-workers of the five antigenic sites enabled their localization into three subdomains of BSA: sites 1 and 4 in subdomain 3 (a.a. #115-184), sites 2 and 5 in subdomain 6 (a.a.#307-385) and sites 1 and 6 in subdomain 9 (a.a.#505-582) (97). It was found that responses to these subdomains increasingly cross-reacted with time. The sites were considered to be in two related groupings a) sites 1-3 and b) sites 4-6, which would explain the cross-reactivity between the domains (93). Mice were primed with these three subdomains and the T-proliferative response was assayed. The conclusions obtained support the proposition that the cross-reactivity seen at the B-cell level is duplicated at the T-cell level (98).

Benjamin’s group has managed to map the response to BSA to the MHC, high responders are H-2^k,a,d and H-2^b are low responders (99). Use of recombinant strains mapped a major gene to I-A for both antibody and proliferative responses with involvement of I-E/C subregion genes. Domain III seems to be the immunodominant determinant in low responders and in the early response of high responders; reactivity to it disappears by the 3° response in high responders.
Thus, conventional antibodies have demonstrated the presence of at least 6 antigenic determinants, which are under H-2 linked immune response gene control, however, the use of monoclonal antibodies has defined 16 distinct antigenic determinants (100), indicating that the above interpretations definitely are only applicable to immunodominant determinants. Despite these complications, recent immunoregulatory studies have been undertaken. A pepsin digest peptide (8,000 – 10,000 m.w.) of BSA was isolated and was shown to stimulate in vivo a suppressor population as pre-immunization with it prevents anti-BSA (IgE) antibody formation. The peptide on its own does not induce antibody production to itself, nor does it inhibit binding of anti-BSA antibody to BSA in vitro (101). Further studies of Benjamin's group are under way and the indications so far are that the domains of BSA are antigenically distinct at the T-cell level, with two or more T-cell determinants per domain, (102) again contradicting earlier observations (98).

2. Collagen. The collagens are naturally occurring structural proteins composed of repeating tri-polypeptide polymers of glycine-led triplets with an extremely high content of proline, hydroxyproline and hydroxylsine. The molecules are in the shape of rigid triple helices. The T-independent (103) murine antibody response to native collagen has been shown to be specific for conformational determinants (104) in a similar fashion to the response to the previously discussed synthetic polypeptides (T-G-A-Gly)n and (T-A-G-Gly)n (59). The response to denatured collagen (gelatin) is T-dependent (103).
A number of different collagens have been studied so far with respect to their immunogenicity in mice with subsequent genetic mapping: a) the response to calf skin collagen type I was originally mapped to I-A in high responder b, f and s haplotypes with evidence for non-H-2 gene involvement (105); subsequently, evidence was uncovered that two or more Ir genes with distinct functions are involved in the immune response to type I collagen (106), b) the genetic control of the T-proliferative response to denatured beef type II collagen maps to the I-A subregion with the H-2^b haplotype; the study demonstrated that the proliferation is dependent on the recognition of primary amino acid sequences, not secondary or tertiary configurations, with at least two antigenic determinants being proposed: Gly-Pro and Pro-Gly (107), and c) the genetic controls to human collagens type III and V (AB^) are distinct from the ones described in a and b above: the anti-type III response maps to I-A/B in H-2^s mice while two gene complementation in I-A and I-E is observed in the response to type V (AB^) collagen (108).

Another interesting line of investigation has centered around the study of procollagen, which consists of the collagen molecule and a globular procollagen peptide. It was found that as the responses to the two components of procollagen were under separate genetic control, immunization of some but not all peptide or collagen non-responders with procollagen could alleviate the non-responsiveness (109). It was also found that preimmunization of collagen non-responder/procollagen responder strains with collagen, suppressed the response, indicating an intricate control to the molecule. Further studies described the dependence on
tertiary structure of antigenic determinants of procollagen (104). The major importance of the studies of the immune response to collagen is the eventual understanding of the clinical disorder, rheumatoid arthritis.

3. Cytochrome c. The amino acid sequence of about 90 cytochromes is known; thus the analysis of quantitative cross-reactions provides a tool to the immunologist to define the specificity of antigen recognition by both T- and B-cells: the original studies describing antibody specificity inferred that antigenic determinants were limited to sequences not shared between the immunizing and host cytochromes and that lack of cross-reactivity between two immunizing cytochromes would again hinge on the sequences not shared between the two; these differences were always found in one of four sequences: I #11-15, II #44-50, III #58-62, 83 and IV #89-92 (110,111). With the use of immunoabsorbents, 'determinant' specific antibodies were produced and further delineation of the residues involved in immunogenicity was obtained with evidence that not all amino acid differences were responsible for the induction of antibody production, yet a single amino acid substitution was shown to affect the antigen binding capacity of specific antibody (110, 112,113, 114, 115).

The determination of T-cell specificity awaited the development of reliable assays. Two competing groups described proliferation assays: 1) Schwartz et al. (116) described the use of non-adherent peritoneal exudate, T-lymphocyte enriched cells (PETLES) in antigen specific proliferation determined by $^3$H methyl-thymidine uptake, and 2) Corradin et. al. (117) described a more simple, reliable assay with the use of
whole lymph node cell populations from selected sites in describing essentially similar T-proliferative restrictions. Using the PETLES assay, T-lymphocyte proliferative responses to pigeon cytochrome c were shown to be under Ir gene control mapping to two subregions of the MHC, the \( \kappa \) and \( \alpha \) alleles demonstrating high responder status while \( b, d, p, q, s \) and \( u \) haplotypes were low or non-proliferators, the specificity of the mouse T-cell proliferative response to pigeon cytochrome c was localized to CNBr fragment 81-104 (118). Further studies by the same investigators expanded the list of non-responding haplotypes by \( f, j \) and \( r \), defined mapping the response genes to I-A and I-E/C and localized the specificity within the topographic determinant to residues \#3, 100 and 104 (119). Immunization with CNBr peptides of pigeon cytochrome c resulted in T-proliferation to only peptide 81-104 (119); however, when a C-terminal 8 residue peptide was synthesized, it was shown to be ineffective in stimulating T-proliferation, indicating that a topographical determinant may be recognized (120).

Corradin's laboratory confirmed the exquisite single amino acid discrimination of proliferating T-cells with the use of closely related cytochromes and CNBr peptides (peptides 1-65, 66-104 and 81-104 were obtained by cleavage at the two methionine residues at 65 and 80) (121). More recent studies by these investigators uncovered two important facets of murine T-cell proliferation to cytochrome c. In the earlier study (122), it was demonstrated that cells from a strain of mice which could not proliferate to native beef cytochrome c nor to the 1-65 CNBr peptide, could exhibit proliferation to the 81-104 peptide, indicating that
phenotypic non-responsiveness may in fact mask populations capable of recognizing certain sequences of the molecule: responsiveness is inhibited by the presence of sequences imparting suppression. In the second study (123), genetic differences were observed in the response to native and denatured forms of beef cytochrome c: macrophages from one parent and the $F_1$ could present both forms of cytochrome c while the other parental macrophages could only perform in the proliferative response when native cytochrome c was used, indicating that differential antigen processing capabilities are involved and genetically encoded.

Evidence for the determination of fine specificity of the response by antigen presenting cells was obtained with the use of T-cell clones originated in two strains of mice which demonstrate different antigen specificity (124). It was found that the clones from one strain, when presented with antigen in conjunction with APC’s from the other strain, exhibited a specificity pattern of the strain providing the APC, suggesting that the specificity repertoire of T-cells reflects the interaction between antigen and MHC gene products of the APC. The cross-presentation phenomenon was re-examined between clones of T-cells which discriminated between single amino acid substitutions (125). Further exciting descriptions of immunoregulation can be expected in the cytochrome c system by these researchers.

In recent studies, with beef cytochrome c, Atassi has suggested that with antigens which have counterparts in the immunized animal, the responses are not directed to sequences not shared between the antigen and the autologous molecule, but that antigenic sites are inherent in their
configuration (126). Three of the four shared sequences described above were synthesized: (a) 42-50, (b) 57-64, and (c) 87-94. Since only peptide 42-50 actively absorbed any rabbit anti-beef cytochrome c antibodies, this was considered a chance event with support of their theory. As well, autoreactive antibodies generated in the rabbit were found to bind this same peptide, supporting the theory of configurational determinants. Unfortunately, as this study was undertaken with beef and not the pigeon cytochrome c used in other studies, comparisons between the various systems cannot be made but may in fact explain the different patterns of determinant immunodominance. However, it may be postulated, that if Atassi is right, and it is the configuration and not the actual sequence differences that determines antigenic sites, the variation between his observations pertaining to B-cell antigen recognition, and the observations pertaining to T-cell proliferation of other researchers, in fact describe a difference between T-and B-cell repertoires.

4. Fibrinopeptide. Human fibrinopeptide B is a 14 amino acid thrombin-derived fragment of fibrinogen which does not induce antibody production in guinea pigs strains 2 and 13, but does induce both DTH as well as a T-proliferative response in strains 2 and (2 x 13)F₁ guinea pigs but not in strain 13 (127). Thus, studies with the goal of determining antigenic recognition by T-cells were undertaken with the use of a number of synthetic homologues of fibrinopeptide: BB 1-14 primed T-cells were tested for proliferative capacity to BB 3-14, 5-14, 7-14, 1-13 and 9-14. Strain 2 and (2 x 13)F₁ T-cells proliferated to the first three but not the fourth and fifth. When these peptides were used
to immunize guinea pigs, BB 1-14, 3-14, 5-14 and 7-14 were immunogenic in strain 2, and non-immunogenic in strain 13, BB 9-14 was non-immunogenic in both strains, while BB 1-13 which was non-immunogenic in strain 2 was immunogenic in strain 13, indicating that non-responsiveness to human fibrinopeptide in strain 13 is due to a binding site which cannot accommodate arg\textsuperscript{14}. An alternative interpretation, not presented by the authors, involves the generation of a suppressor determinant. The involvement of a defect at the APC level was postulated.

Further studies (128) using single amino acid substitutions of the peptide BB 5-14(NH\textsubscript{2}-Asp\textsuperscript{5}-Asn\textsuperscript{6}-Gln\textsuperscript{7}-Gln\textsuperscript{8}-Gly\textsuperscript{9}-Phe\textsuperscript{10}-Phe\textsuperscript{11}-Ser\textsuperscript{12}-Ala\textsuperscript{13}-Arg\textsuperscript{14}-OH) demonstrated that Arg\textsuperscript{14} plays a major role in the specificity of the response, as substitution by lysine produces an immunogenic peptide that is not cross-reactive at the proliferative level. Other substitutions at residues 5, 7, 9, 10 and 11 produce analogues which have a spectrum of reactivities from unaltered to totally non-cross-reactive. Postulates as to the nature of the Ia-antigen complex recognized by T-cells are put forward and then more actively studied in a following publication (129). BB 7-14 and the inverted peptide BB 14-7 were used to assay the fine specificity of T-cells; it was found that BB 7-14 T-cells would not proliferate to BB 14-7 nor was BB 14-7 immunogenic, indicating that polarity of the molecule is important. Formation of neoantigenic determinants in the association of Ia molecules was postulated. BB 7-14 which is non-immunogenic in strain 13 guinea pigs was found to become immunogenic with the addition of Gly at the COOH terminus, the suggestion being, that attachment to self Ia was facilitated. It is
evident that more stringent analysis will have to be performed in order to confirm the above hypotheses.

Recently, Peterson et al. (130) have undertaken the study of the murine anti-fibrinopeptide B response at the T-proliferative level. Synthetic BB 1-14 immune H-2^k purified T-cells demonstrated a good proliferative response to soluble BB 1-14, whereas the h, d and g haplotypes were shown to be non-proliferators. This response was found to be dominant in (R x NR)F_1 and maps to the left of I-C. Although no antibody to BB 1-14 was detectable, more in-depth analysis localized the T-cell determinant to the terminal 8 amino acids, i.e. 7-14, corroborating the guinea pig studies. Both investigations, in the guinea pig and murine system, are rather elegant and deserve further attention.

5. B-galactosidase. B-galactosidase (GZ) is a large tetrameric molecule that has been used extensively in studies of immunoregulation not pertaining to Ir gene control. The initial observations using GZ in a hapten carrier, TNP-GZ (131), and adoptive transfer systems (132), were that T-cells mediate successive time, cell number and dose dependent waves of suppressive and helper functions. This pattern of response was further dissected using CNBr peptides of GZ in order to separate determinants providing help from those providing suppression (133). It was found that the peptide CB-2 (a.a. 3-92) imparts a suppressive function in the hapten (FITC)-carrier response that is not preceded by the normal stimulation of helper functions observed with the native molecule indicating that the balance observed in the response to the native molecule may be dependent on the presence of suppressor and helper determinants located on different
parts of the molecule.

In order to determine the T-helper repertoire, eleven CNBr peptides of GZ, covering 70% of the molecule, were generated and tested for their ability to induce *in vitro* proliferation and help in the hapten-carrier system, FITC-GZ (134). Five of the peptides demonstrated strong priming for the proliferative response, four were intermediate and two (CB-20 and CB-4) lacked this capacity. These data point out two salient facts about T-cell repertoires and functional T-cell assays: 1) T-cell proliferation is not an absolute reflection of help as T-helper cells have been demonstrated to have a much more restricted repertoire in the FITC-GZ response, 2) the previous postulate that CB-2 provides exclusively T-suppressive functions, while other regions of the GZ molecule provide help which is modulated by the CB-2 region, has been disproved in a later publication since CB-2 was shown to be one of the two regions capable of demonstrating help in the FITC-GZ response. This dichotomy could be explained by the presence of two separate determinants on CB-2, one for help and one for suppression, or alternatively, that help and suppression were induced by the same sequence. On the other hand, the investigators brought up the possibility that poor fluoresceination of GZ (17-25 FITC per GZ molecule) could account for the limited T-helper repertoire. Whatever mechanism is involved, more in-depth studies will have to be performed (possibly dealing with peptides of smaller size) in order to elucidate the T-helper and T-suppressor repertoires - are they shared or distinct?
6. **Glucacon.** The 29 amino acid hormone, glucagon, was used in a study with guinea pigs in order to assess humoral versus cellular specificities in an immune response (135). All animals tested produced antibodies specific for the amino-terminal peptide (1-17) generated by tryptic digestion of the molecule with no detectable binding to the carboxy-terminal peptide (18-29); the smallest synthetic peptide binding the antibody comprised residues 5-16. DTH and MIF assays were used to assess cellular immunity; the activity seen to the native protein was mimicked by the C-terminal peptide and a synthetic molecule comprising residues 19-29. Neither of the products of trypsin cleavage were immunogenic on their own. This simple molecule provides the unit requirement for T – B collaboration.

7. **Haemoglobin.** With the increasing understanding of the genetic control of simple protein antigens, attention has recently shifted to the study of immune responses to more complex antigens, such as β-galactosidase described above, in order to ascertain the patterns of responsiveness to oligomeric proteins. Human adult haemoglobin (Hb) is a tetramer of two pairs of non-identical subunits, α and β, and was used in a series of studies to elucidate the role of individual subunits in the regulation of the immune system.

Initially, the immune response was studied using the intact Hb molecule to immunize congenic strains of mice, followed by testing the response via the proliferation assay with Hb as well as the individual α and β subunits (136). This study demonstrated that the b and d alleles were responsive to Hb, while j, k, g, ε, and s were low proliferators to
Hb. When the α and β specific responses were tested and subsequently mapped with the use of recombinant strains, the suggestion was made that the response mapped to the I-A for the α subunit and the D-end of H-2 for the β-subunit. However, it was not until the subsequent publication (137), that the response to each determinant was studied more conclusively following immunization with the subunits as opposed to the intact molecule: separate alleles of responsiveness were found to the individual subunits (as seen in Table I), yet both responses were found to map to the I-A subregion; the α-subunit response was further associated with the la.8 specificity with the use of mutants (138). Although the responses map to the same subregion for the two subunits, the proliferation induced by either was not cross-reactive (137); interactions in the responses to the individual subunits were postulated to affect the response to the native molecule. In subsequent work, non-H-2 effects were studied in the response to the subunits of Hb; non-H-2 effects were found for the T-proliferative responses to both the α and β subunits (139).

8. **Hepatitis B antigen.** One of the by-products of the types of genetic investigation discussed in this chapter is the application of technology accumulated with experimental antigens to the study of antigens associated with disease (such as collagen described above). This can be best exemplified in the search for a synthetic vaccine to the hepatitis B antigen: localization of the determinant responsible for immune reactivity could lead to the synthesis of fairly simple synthetic immunogenic peptides (140). An observation that sequence-dependent antigenic determinants are often found in hydrophilic regions of protein
antigens (141) prompted the investigators to look at the hydrophilic regions of the $\alpha$ determinant (serologically determined to be the major determinant seen by the human immune system). Sequences 32-76 and 110-156, rich in hydrophilic residues, were studied coupled with investigations concerning the involvement of lysine in immunogenicity (the second sequence is lysine rich). As chemical modification of lysine eliminated immune reactivity to the antigen, 110-156 sequence analogues were synthesized in order to size the antigenic determinant: 139-147 was found to represent the essential residues of the $\alpha$ determinant. This study elegantly points out a clinical application of immunochemical studies.

9. Insulin. Mouse and guinea pig anti-insulin responses have been extensively studied during the last decade as insulin provided a small, well-defined antigen with biological variants that could enable the assignment of highly specific sequences to determinants recognized by T-cells in Ir gene controlled cellular interactions. Keck's pioneering studies in the mouse system demonstrated that residues A6 to A11, comprising the disulfide-bridged A-chain loop, provided one of the determinants recognized by T-helper cells in a response to DNP-substituted bovine or pig insulin, depending on the mouse strain (142). Subsequent studies with bovine insulin, which differs from mouse insulin at 5 amino acid residues, tentatively mapped the response gene(s) to I-A and extended the list of responder/non-responder strains (see Table I) (143). The response patterns were found to be dependent on the mode of immunization (B. pertussis versus CFA) and antigen dose. (Subsequent studies by other
investigators confirmed the importance of immunization protocol (144), see below). Other preliminary studies which were also confirmed by other investigators (see below), indicated that non-responder mice could be made to respond to insulin when immunized with proinsulin but not when insulin was linked to a carrier protein such as bovine or rabbit IgG or methylated BSA. Further genetic analysis of the anti-insulin response indicated that complementation to produce high responses was observed in certain, but not all crosses of non-responding animals (145). The presence of three genes was postulated by rather complicated reasoning: two in H-2 and one on the C3H/He background. They concluded that multideterminant responses were measured (where non-responding parental strains each recognized different hidden determinants and the responder F1 produced a response by recognizing both determinants); this hypothesis was tested by co-immunization with no clear results.

Rosenthal and co-workers have provided a large body of information on the immunoregulation of the response to insulin in guinea pigs as well as mice. In the first study using guinea pigs (146), T-proliferative and T-helper assays identified amino acids A8, A9 and A10 of the A-chain loop as the determinant recognized by both the proliferating and helper T-cells of strain 2 guinea pigs, while the response to oxidized insulin B-chain was exclusive to strain 13. When a proliferating population responding to one chain was treated with BudR and light, very little proliferative capacity to the native molecule was left, indicating that proliferation was indeed restricted to one chain. However, when anti-pork insulin antibodies were tested (pork insulin is immunogenic in both strains), they
were found to share specificities, i.e. strains 2 and 13 produced cross-reactive antibody of very restricted heterogeneity despite the fact that each strain recognized different determinants/chains, at the T-cell level. Further experiments described Ir gene control to be functioning at the level of macrophage determinant selection (147).

The above results in guinea pigs were found to be duplicated in the mouse system (148): in the T-proliferative response, two responder strains were found to recognize different determinants. H-2<sup>b</sup> mice recognize the A-chain loop while H-2<sup>d</sup> mice are specific for the terminal eight amino acids of the B-chain. Both these responses were found to map to I-A, with non-H-2 control implicated. It was proposed that Ir gene regulated determinant selection occurred at the level of the macrophage (148,149). Subsequent studies by another investigator employing in vitro stimulation of T-helper cells with genetically related macrophage factor to insulin, confirmed the mapping to the I-A as well as emphasized that the Ir genes function at least at the level of the macrophage (150).

In another study using an insulin-SRBC plaque assay, it was demonstrated that non-responsiveness was mirrored by lack of anti-insulin IgM or IgG antibody, while insulin responding strains made 50% IgG1 and 50% IgG2 antibody (151). With the use of synthetic polypeptides it was demonstrated that residue 10 (a histidine) on the B-chain is critical for guinea pig T-cell activation as measured by proliferation and is dependent on macrophage recognition of this residue (152). A hypothesis was put forward that macrophages process antigen and present fragments containing the necessary T-cell stimulatory sequences, which are predicted to be
distinct from sequences recognized by antibody.

It was established in the guinea pig system, that immunization with insulin in Incomplete Freund's Adjuvant (IFA) induced suppressor cells which could inhibit a proliferative response to insulin/CFA primed T-cells (153). This suppression was subsequently demonstrated to be controlled by immune response genes. However, the determinant previously shown to induce the proliferative response was found to be identical to the one responsible for the suppression of the proliferative response (154). Moreover, this suppression protocol, although it induces a suppressed anti-hapten response, does not suppress the anti-insulin response.

Kapp and co-workers have studied the genetics of the response at the antibody level as well as the basis for non-responsiveness. Isoelectric focusing of murine anti-porcine and anti-bovine insulin antisera demonstrated restricted heterogeneity as compared to guinea pig anti-insulin antisera and antisera to a number of other protein antigens (155). The patterns of the spectrotypes were strain specific and codominant in $F_1$ mice. The triggering of only a few B-cell clones may be due to the few amino acid differences between the autologous and immunizing insulins. Two additional amino acid differences ($A8$, $A10$) between bovine and porcine insulins on top of the four differences between them and the murine insulin ($A4$, $B3$, $B30$, $B29$) account for the cross-reactivity in anti-bovine and anti-porcine antibody; the "leftover", non cross-reacting antibody to bovine insulin reflects the additional $A8$, $A10$ difference between the two insulins.
In a related yet conceptually conflicting study, other investigators commented on the fact that immunization with these closely related insulins results in a degree of autosensitization due to intramolecular cooperation between different determinants on the same molecule (144). The occurrence of autoreactivity would suggest that all available B-cell clones could be triggered to produce antibody. In order to reconcile these two reports (144, 155), three possibilities may exist: 1) autosensitization occurs under stringent conditions not employed by the first investigators, 2) the anti-insulin clonal repertoire is small and comprises both self and non-self reacting clones yielding low heterogeneity in the spectrotypes, or 3) regardless of the insulin variant used, a very small number (e.g. only 2) of determinants are present on the insulin molecule, the determinants being shared between the immunizing and autologous insulins, thus, any response is to the same (two) determinants. (This subject will be discussed later in more detail in reference to other antigenic systems).

Kapp et al. further set out to analyze the nature of non-responsiveness by expanding on the system introduced by Keck: the use of proinsulin to induce non-responders to generate an anti-insulin antibody response (156). It was found that some (H-2\(b,k,s\)) strains were successfully immunized with proinsulin while others (H-2\(q\)) were not, indicating that proinsulin (M.W. 9000) is under Ir gene control as well. It was, however, surprising to find immunization of proinsulin (R x NR)\(_{F1}\) mice with proinsulin produced no anti-insulin antibody, indicating that despite the fact that proinsulin binding antibodies are generated in
the F₁, a defect in the insulin-specific response exists (a gene dose effect or active dominant suppression were postulated). Thus, two types of phenotypic non-responsiveness are seen in the insulin system.

Another very interesting phenomenon was the recent finding that pork insulin non-responders (non-responders do not proliferate nor make anti-insulin antibody), when primed with the non-immunogenic pork insulin and subsequently challenged with an immunogenic insulin (beef), demonstrate that there is a population of T-helper cells stimulated during the non-antibody-productive primary immunization (157). Through adoptive transfer studies, a two T-cell model was proposed. In a similar but somewhat more complicated approach, the above observations were confirmed in a study of the control of the murine T-proliferative responses to a panel of insulins, in order to pinpoint antigenic determinants: a complex gridding of responses indicated that immunization of a strain with a "forbidden" (i.e. non-immunogenic) insulin primed for an in vitro response to a "permitted" (i.e. immunogenic) insulin (158).

Further preliminary studies using a different handle in the study of non-responsiveness have centered around the use of a T-independent form of insulin: insulin chemically coupled to the T-independent organism Brucella abortus (159). It was found that regardless of the responder status to insulin, both responder and non-responder strains produced IgM and IgG anti-insulin antibodies to T-independent insulin. The ensuing studies from these observations will hopefully provide interesting insights into cellular interactions.
10. **Lactate dehydrogenase B.** The response to lactate dehydrogenase B (LDH\(_B\)) was originally mapped to I-\(B\) and I-\(C\) (160,161), however, at a later date, the validity of I-\(B\) subregion control, a subregion that has no serologically detectable gene product, was contested. As addition of anti-I-\(A\) monoclonal antibody to T-cell proliferation cultures converts non-proliferators to responders to LDH\(_B\), the control was postulated at the level of complementing genes between I-\(A\) and I-\(E\) (162). LDH\(_B\) has been used as a probe for examining cellular interactions in suppressor pathways (163); however, these studies are beyond the scope of discussion within this thesis.

11. **Lysozyme.** Of all naturally occurring antigens used to study immunoregulatory functions, lysozyme has proven to be one of the most rewarding molecules despite its exquisite dependence on native configuration. It was established originally in our laboratory (164,165), and subsequently confirmed elsewhere (166), that anti-native versus anti-reduced, s-carboxymethylated lysozyme antisera are totally non-cross-reactive. When T-proliferative responses were assayed, using both types of lysozyme, absolute cross-reactivity was demonstrated, regardless of the degree of denaturation of the proteins; the investigators correctly deduced at the time that T-cells "see" antigen as a result of macrophage processing which degrades both forms of lysozyme to the same end-product, while B-cells react to the unprocessed antigen (166). Selective processing (degradation and elimination versus interaction with self and presentation) by macrophages of antigenic determinants was postulated to result in Ir controlled selection between
suppression or help of an immune response (167). Subsequent studies with chimeras have limited non-responsiveness due to suppression at the level of the antigen presenting cell and the T-cell in the anti-lysozyme response (168).

The major questions addressed with the lysozyme molecule have been those of immunoregulation by Sercarz and co-workers with emphasis on the balance of T-suppressor and T-helper repertoires (169) and the interactions in idiotypic and antigen bridge-dependent networks (170). Other workers have studied the immunochemistry and genetics of the response to lysozyme. This second aspect of the research will be dealt with first and will be followed by the sophisticated immunoregulatory studies.

In a painstaking series of investigations (reviewed in 171, 172), three antigenic determinants of hen egg white lysozyme (HEL) for B-cells were proposed and synthetic molecules produced that were considered to most closely resemble the tertiary configuration (topographically) of the determinants:

- **site 1)** \( \text{arg}^{125}-(\text{gly-gly})-\text{arg}^5-(\text{gly})-\text{glu}^7-(\text{gly-gly})-\text{arg}^{14}-\text{lys}^{13} \)
- **site 2)** \( \text{trp}^{62}-(\text{gly})-\text{lys}^{97}-\text{lys}^{96}-\text{asn}^{93}-\text{thr}^{89}-\text{asp}^{87} \) and
- **site 3)** \( \text{lys}^{116}-\text{asn}^{113}-(\text{gly})-\text{phe}^{34}-\text{lys}^{33} \) (in subsequent work by Sercarz's group, site 1 forms part of the N-C molecule while sites 2 and 3 are included in the L_{11} fragment). Okuda et al. (173) have mapped the response to I-A and tentatively to I-C with recombinants of the high responding f, k, d and p haplotypes and low responder b, g and s alleles.
Studies by Sercarz and co-workers (174) were in agreement with the above genetic assignments and provided further information on the genetic control of the anti-lysozyme response: 1) not only was it found that indeed both I-A and I-C subregions were involved in the regulation of the response but also that possession of either locus was sufficient in most cases and 2) whereas the non-responder status is a recessive characteristic linked solely to H-2, high responder genotype is affected by non H-2 genes which control the magnitude of the response in a co-dominant fashion with the H-2-linked genes. This non-H-2 control was reported on in a further publication (175), where it was determined that the primary IgG response is not affected by these genes. It is not until the secondary response that 25-fold differences are observed in the antibody response between A/J and B10.A mice, where the B10.A response is actually much lower than its primary. It seems that the A/J response is much more heterogeneous when isoelectric focusing is performed on ascitic antibody. Although the non-H-2 control over magnitude is co-dominant, the heterogeneity of the (A/J x B10.A)F₁ antibody population is that of the A/J parent.

As with cytochrome c, insulin and myoglobin, lysozyme is present biologically in an array of species variants which in all cases share considerable homology. This fact was capitalized on in the early studies on the prototype HEL with mapping of its immune response gene, Ir-GEL; the fine specificity of this Ir-GEL locus was determined at the level of discrimination of a few amino acid differences between HEL and nine other lysozymes (176,177). All strains but H-2 (and s) were found to be
responsive to a panel of lysozymes, whereas $H-2^b$ animals were found to be non-responsive to HEL as well as human, bobwhite, Gambol quail, Valley quail and guinea hen lysozyme, high responders to Japanese Quail and ring-necked pheasant lysozyme (REL), and intermediate responders to turkey and peafowl lysozyme. There was a high correlation in the $H-2^b$ to the presence of phe at residue three in the non-immunogenic lysozymes as compared to tyr at this position for immunogenic lysozymes; however, intermediate responder status was attributable to other differences in the antigen molecule. With the two intermediate lysozymes there exists a dichotomy in the responses they induce, which show poor antibody production but a strong proliferative response.

HEL coupled to LPS was used to immunize non-responder ($H-2^b$) mice in order to assess the presence of memory B-cells in these mice (178). An HEL-primed B-cell (in B10.A mice for example) would be triggered by an HEL-LPS signal to produce an IgG response, however, $H-2^b$ mice were only capable of mounting an IgM response following this immunization protocol. It was concluded that the T-helper cell dependent IgM-IgG switch is absent in the non-responding strain, presumably due to a T-suppressor function.

The non-immunogenic lysozyme (HEL) when coupled to a carrier (RBC) was found to induce a response, indicating that the lesion involves the lack of appropriate T-helper cell function (179). Pre-immunization with HEL suppressed this response; this suppression was reproduced in vitro with an I-J$^+$ T-cell that was specific for the HEL response and affected both IgM and IgG production of only anti-HEL antibody but not antibody to the closely related REL. These results suggested that a sequence of
residues unique to HEL but not REL could constitute a suppressor determinant; studies were carried out to see whether this determinant could be eliminated.

A reduced and carboxymethylated CNBr fragment, $L_{II}$, was used to assess the presence of a suppressor determinant comprising residue three (180). The non-responder C57BL/10 ($H-2^b$) and responder B10.A ($H-2^a$) mice were immunized with $L_{II}$; they were shown to be primed for a good proliferative response in both cases whereas the intact HEL molecule stimulated good proliferation only in B10.A. Mixture of HEL-and $L_{II}$-immune PETLES in a 1:50 ratio was sufficient to demonstrate susceptibility of the responding cell to suppression induced by HEL immunization, indicating that in $H-2^b$ mice, although the potential for a response exists, the induction of suppressor cells is a superceding event.

The suppression induced by the native HEL was analyzed with the use of a peptide generated by mild acid hydrolysis: it has an intact disulfide between sequences 1-17 and 120-129 in order to maintain partially its configuration (181). Pre-immunization with this N-C peptide was found to duplicate the inhibition of the HEL-RBC response in non-responder mice whereas it was capable of stimulating help in the responder B10.A strain.

More recent studies regarding the specificity of the T-proliferative response using limiting dilution analysis have confirmed in the responder B10.A strain that $L_{II}$ (aa 13-105) is the major determinant inducing T-cell proliferation, equalling the proliferation induced by the native molecule (182). Lower proliferative activity was seen with N-C and a low
but significant response was seen with the CNBr peptide $L_{II}$ (106-129). Via cross-stimulation studies, the overlapping region between $L_{II}$ and $N-C$ (aa 13-17) as well as a sequence from 106-121 seemed critical in inducing proliferation, while residues 120-129 were again shown to have little stimulatory function. The non-responder B10 strain was incapable of responding to either 13-17 or $L_{III}$ in vitro, indicating that a second non-responder mechanism (besides the N-C inducible suppression) is involved in the HEL non-responsiveness in the B10 mouse. It was, however, proposed to be independent of active suppression, which is a function of immunizing dose: non-responsiveness to 13-17 was found not to be dose dependent, hence proposed to be a separate non-responsiveness phenomenon (183).

With respect to genetics of the proliferative response, preliminary interesting insights have been obtained: the previously reported results (174) mapping the antibody response to both I-A and I-C were shown to hold true for the proliferative assay (184). In addition, determinant selection is exhibited by these two loci: residues 113-114 (N.B. part of Atassi's site 3) are singularly immunogenic in $H-2^d$ mice, whereas other regions cross-reactive between the two lysozymes, HEL and REL, are preferentially immunogenic in B10.A and B10.A (5R) mice.

In-depth analysis of this phenomenon as well as other immunoregulatory facets of the response to lysozyme are eagerly awaited.

Evidence for the presence of a T-helper population could also be elegantly demonstrated in vivo in non-responder mice, even without the use of immunization with $L_{II}$, but to a HEL stimulus: helper activity was
found early (4 days) in the response in local draining lymph nodes, comparable to immunization with REL, as opposed to a generalized suppression seen in the spleen and later (21 days), in the same draining lymph nodes (185). It was demonstrated that early in the response helper cells were induced in the lymph nodes as well as an early $I-J^+$ suppressor cell inducer. Later in the response, a second $I-J^-$ suppressor precursor cell type was shown, in mixing experiments (10 day + 21 day lymph node cells), to recruit the first appearing suppressor cell type to produce suppression.

Evidence for the presence of a helper cell population can also be demonstrated in B10 mice with careful analysis of the proliferative response (170): it seems that HEL-immune B10 cells, when put in a PETLES assay, demonstrate a low (about 10% of the PPD response), but reproducible proliferation when challenged in vitro with HEL, and a six times higher response with REL, indicating that priming with HEL has induced a helper function that is almost totally masked in the non-responder animal via the activity of the suppressive functions. This result was interpreted to mean that two helper populations are present: one that is induced by HEL priming and seen in the proliferation when the suppressor determinant was missing (i.e. probably localized within $L_{II}$), and a second recognizing sequences in close association with the suppressor determinant. The second helper determinant is the one responsible for the proliferation seen to HEL in HEL-primed B10 PETLES described above; its location has been mapped to residues 84 and 92 on the N-C peptide by two lysozyme variants which could cross-reactively proliferate with HEL primed B10.A
but not B10 PETLES. Amino acids 84 and 92 are the only two common residues on the two lysozymes but absent in all others. Evidence for these two helper T-cells was sought in a more direct fashion in the responder B10.A mouse using HEL immune T-cells to adhere either to HEL or idiotype coated plates (170,186). The two thus separated populations, inactive on their own, were found to reconstitute the response to HEL: one population with specificity for a response via the antigen bridge, the other implicated in an idiotypic network, both being needed for the HEL response.

The anti-HEL response in B10.A mice was found to be almost entirely directed to the N-C peptide (this was not true of other H-2^a strains on a different background e.g. A/J) (187). Anti-idiotypic serum used in the above studies (170,186), showing a presence of Id^+ B-cells, was generated in guinea pigs to purified ascitic antibody from HEL-immune B10.A mice (188); this antiserum was additionally used to determine that the suppressor cells described in the B10 HEL-RBC assay (179), are idiotype positive. However, when B10.A helper cells are treated with anti-idiotypic plus complement, no effect is observed, indicating that antigen specific T-helper cells do not bear IdX.

More recently, with the advent of hybridoma technology, it was found that idiotypes expressed on monoclonal antibodies, often yield fairly interesting aspects of immunoregulation that could not be elucidated with the use of heterologous antibody induced anti-idiotypic sera. However, it seems now that some rather perplexing facts are coming out concerning antigenic specificity of heterologous versus monoclonal antibody. A
recent example is found in the study of anti-HEL monoclonal antibodies (189): it has been shown above (187,190), that 70-95% of the anti-HEL response in B10.A is reactive with the N-C peptide with a predominant idiotype, IdX-HEL. The small proportion of $L_{II}$ reactive antibodies was found to be IdX-HEL negative. Other workers (191) studying this loop specific antibody have found that residue 68 is strongly implicated in the binding of anti-HEL antibodies with $L_{II}$. When monoclonal antibodies (A/J origin) were generated to HEL, some were isolated with affinity to $L_{II}$ while others showed N-C affinity (189). A monoclonal antibody, AD2, not demonstrating appreciable binding to either $L_{II}$ or N-C, was used to produce anti-IdX-HEL antiserum which was shown to have high specificity for anti-HEL antibody as compared to antibody to essentially non-cross-reactive lysozymes. It was found that this antiserum could totally inhibit a $L_{II}$ specific (thus IdX$^+$) as well as an N-C specific monoclonal (IdX$^+$) antibody, whereas a third monoclonal (IdX$^-$) was not inhibited, indicating that a common IdX-HEL marker is present on some hybridoma products of different determinant specificity but to the same antigen. These hybridoma products were capable of absorbing all the activity from the anti-IdX-HEL antiserum and from the antiserum to conventional anti-HEL antibody.

The IdX-HEL marker does not appear until later during an immune response (192), indicating that the predominance of IdX-HEL is a result of positive selection for this idiotype and may explain the presence of this idiotype on T-cell subpopulations (T-suppressor): IdX-HEL$^+$ - recognizing T-helper cells were shown to interact with antigen-specific T-helper cells
in the antibody response to HEL (186). This close interaction between idiotype and antigen specificity may explain why this idiotype was found on L\textsubscript{II} and N-C specific monoclonal antibodies.

So far, the lysozyme system has provided us with some elegant models of immunoregulatory interactions between suppressor and helper T-cells, and B-cells via both antigen bridging as well as idiotypic control. Future research with this molecule is apt to complete the picture of immunoregulatory interactions.

12. Myoglobin. Immunochemical studies on the prototype myoglobin, sperm whale myoglobin (Mb), were begun in the 1960's (193) in Atassi's laboratory. By 1975 (194), the antigenic structure had been resolved and the molecule was divided into 5 antigenic regions: (1) residues 15-22, (2) residues 56-62, (3) residues 94-99, (4) residues 113-119, and (5) residues 145-151. These sites did not coincide with amino acid differences between the sperm whale and host myoglobin (rabbit and goat were tested), while antibodies to Mb were found to cross-react with the rabbit myoglobin, implicating the involvement of autoreactive clones (195,196). In fact, immunization of rabbits with homologous myoglobin did induce measurable autoantibodies (197). The suggestion was made that the conformation of certain sequences was important in antibody binding and that the same unique conformational sites induce responsiveness in all animals as the response to myoglobin was found to be independent of the immunized species (198).

When sera from a rabbit were tested from the time of appearance of antibody (9 days) until one year for the specificity of the antibody (via
cross-reaction of 13 different myoglobins) it was discovered that after an initial period of fluctuation, immunodominance was established and the same reactivity profile maintained indicating that the topographic determinants that are recognized are a structurally inherent phenomenon of the molecule (199). Additionally, the antibody affinity was found to be no different between variant strains as assessed by the binding of antibodies to an array of myoglobins (200).

In the original effort to assign B-specific and T-specific determinants on Mb, peptides representing the above 5 regions, as well as the amino-terminal hexapeptide, were utilized to study Mb-primed rabbit lymph node cell cultures, production of MIF, IgG synthesis and DNA synthesis (201). Peptides (2) and (5) induced antibody synthesis and all peptides except for the hexapeptide induced MIF production.

Young et al. (202,203) had at the time attempted with little apparent success, to determine the genetic control of the Mb response: gene dose response curves were constructed in order to discriminate the response between different strains, however, a continuum of response was observed, with high responsiveness associated with the k, f and d haplotypes.

At about the same time, Berzofsky published his first studies on the genetic control of the antibody response to Mb: both H-2- and non-H-2-linked genes were implicated (204). Haplotype d and s mice were shown to be high responders and k, b and q were low responders, contradicting the previous studies (203). High responsiveness was found to be dominant (87) and mapped both to I-A and I-E (204); each region
could independently or in conjunction with the other regulate the response (87). At the time, response to the fragment 132-153 (a product of CNBr cleavage and slightly larger than site (5) above) was quantitated in Mb-immune sera and the critical proposal was put forward that each Ir gene controls the response to a different chemically defined determinant (204).

A number of years later, an in vitro antibody production assay was devised in which peptide-specific antibodies could be tested (205) and the in vivo results were completely corroborated: both the H-2 and non-H-2 linked phenomena were reproduced as well as the determinant specificity to be discussed later. The variation in the observations by Berzofsky and Young et al. above, are difficult to interpret as different immunizing doses of antigen were used and the assays were not quantitated in the same way.

Berzofsky's genetic assignment of the response genes was also completely duplicated by Okuda et al (206) in the determination of the T-proliferative response to Mb: both the strain distribution and the presence of two Ir genes were confirmed. In a subsequent study, Berzofsky et al (84), studying both antibody production as well as T-proliferation, investigated the determinant specificity of both of these aspects of the response. The proposal was that since the two Ir genes controlling the response could in most cases regulate the response on their own, different genes controlled the response to separate determinants. It was found that intermediate responding strains could, for example, make antibody and proliferate to one fragment and a high responding strain could respond to an additional fragment. A parallel determinant specificity was found.
between the assays suggesting the T- and B- repertoires to be similar, if not identical.

Okuda et al. (207), using the T-proliferative assay and the five synthetic antigenic peptides, delineated the determinant specificity as follows: haplotypes d, f and s (the previously described high responders) were found to be high responders to sites (1) and (2), whereas only d and s were high responders to (5). It was suggested that the response to site (3) was non-MHC controlled, whereas sites (2) and (4) stimulated Mb non-proliferating strains, \( H-2^b \) and \( H-2^k \). Further mapping studies localized the response to sites (1), (2), (4) and (5) to I-A and sites (1) and (2) to I-C. In a later communication it was pointed out that the chemical properties of the sites had a critical effect on the genetically controlled recognition of the site. Through the use of myoglobin variants, it was shown that the alteration of chemical properties within a site determine whether or not the site is going to induce antibody via genetically controlled mechanisms (208). An additional aspect of determinant recognition rests on the fact that interactions between sites in the induction of proliferation can affect the outcome of the response (209). For example, in certain strains, interactions of help generated to sites (1) and (2) with site (3) yields a response, whereas site (5) was found to be instrumental in suppression in other strains.

In subsequent studies, the question of autoimmunity was re-addressed this time asking whether the autoimmune response in mice correlates with a genetically controlled determinant selectivity (210). The \( H-2^8 \) strain was the only high responder to murine myoglobin, b, d, f, j, k, p, q, u
and v mice were all low or non-responders; recombination studies again indicated two gene control.

A problem has recently arisen with respect to genetic mapping of the response to myoglobin: despite the fact that highly similar topographic determinants are present to a degree on all myoglobin variants, Berzofsky et al. (211) have found an altered control to equine myoglobin as compared to the prototype Mb. With the use of a H-2D/L region mutant strain and conventional recombinant strains, the antibody response to equine myoglobin was mapped to I-A and H-2D although antibody to these myoglobins strongly cross-reacted. These findings may indicate that more genes than the expected number controlling the anti-myoglobin response exist to the right of I-J in H-2; this would, in part, reconcile the fact that T-cell cloning studies map the response to I-E (212), conventional studies map the response to I-C (see above) and now there is additional evidence for genes to the right of this region.

In separate studies, the B-cell repertoire was assessed through immunoabsorbent titration studies: it was found that the total amounts of antibody binding to synthetic peptides representing the five antigenic sites accounted for all anti-Mb antibody (213). This calculation was contradicted in another study utilizing instead of these 5 peptide antigenic sites, CNBr fragments of the Mb molecule (214) (I 1-55, II 56-131 and III 132-153) where the cumulative absorption to the peptides on solid support only accounted for 60-70% of the total antibody with the balance binding only the native molecule. Subsequently (215), through the use of a radioimmunoassay, these peptides demonstrated $10^4$-fold lower
capacity for inhibition of the binding of anti-Mb antibody to Mb; however, antibodies purified on peptide immunoabsorbents were preferentially inhibited by the peptides themselves than by the native molecule, indicating that there exist varied complex populations of antibody reactive with a globular protein antigen such as Mb.

Additional disagreements in the delineation of antigenic determinants on sperm whale myoglobin were encountered with the manufacture of monoclonal antibodies: out of twelve myoglobin-binding antibodies, none bound either 1-55 or 132-155; additionally, cross-absorption studies indicated that these antibodies recognized multiple determinants cross-reactive with either or both, human and horse myoglobin (216).

When comparing T- versus B-cell repertoires in the five determinant system, it was demonstrated that only the sites inducing significant proliferation stimulated antibody production suggesting that the specificity of T- and B-cells was similar (213). This study also confirmed that different species recognized these same topographic but sequential (i.e. not discontinuous, as in lysozyme) determinants.

When T-cell clones were produced (212), the analysis of receptor specificity was continued, however, slightly contradictory results were obtained: a dichotomy existed, for example, in the recognition of peptide 15-22 (part of the CNBr fragment 1-55), when clones reactive to 1-55 did not proliferate to 15-22, yet antibody to 1-55 was found to bind 15-22. The suggestion was made that the above was evidence that T-cells do not recognize antigen in the native conformation or that distinct regions are
recognized by T- and B-cells.

Studies on the nature of topographic determinants has implicated the involvement of residues outside the five sites described above: cross-reactivity between three myoglobins (beef, sheep and pig), which are identical at 15-22 but differ slightly in other regions, indicate that the small differences outside 15-22 between these variants affect the spectrotypes of antibodies to site (1) (15-22) (217).

With the advent of hybridoma technology, idiotypic analysis of the anti-Mb response was undertaken (218): through the use of a panel of myoglobin variants, the specificity of a number of monoclonal antibodies was assigned. Simultaneous binding to Mb by a number of hybridoma products indicates that some hybridoma products recognize different determinants and the prediction was made that these molecules would have distinct idiotypes. In studies reminiscent of those performed with HEL, monoclonal antibodies not sharing determinant specificity were found to share idiotypic specificities in some cases, suggesting that a regulatory T-cell function was being reflected.

Finally, Berzofsky and co-workers have used Mb to dissect some immunoregulatory phenomena at the cellular level: in the study of macrophage (Kupffer cell) restrictions, it was demonstrated that in strains which respond to different CNBr fragments of Mb under differential Ir gene control, the response is directed by the presence of the appropriate Ir gene product for the particular fragment, in the Kupffer cell (219). These results are found to be in contrast with work by Infante et al. (212): genetic low responsiveness in certain cases was
found not be due to a failure of an APC function when T-cell clones were used.

Further studies by Berzofsky and Richman (220) have shown that presentation of antigen on APC in a proliferative response can be abrogated with the addition of anti-Ia antibodies during the antigen pre-treatment of the macrophages. Anti-I-A antibodies were found to inhibit antigen presentation even when the response under study was localized specifically to I-C. Additionally, the anti-I-A treated antigen presenting macrophages were found to suppress proliferative responses when additional macrophages and soluble antigen were present.

Quite recently, in an elegant study, mice tolerant to the MHC of strains exhibiting the opposite responder status were used to confirm the site of the lesion in non-responders (221). The results have shown that both low and high responder T-cells can help only high but not low responder B-cell (and or macrophage) cultures. Further studies are awaited in which the lesion can be pinpointed to one or both of the low responder cell types.

13. **Staphylococcal nuclease.** Immunological studies with staphylococcal nuclease (Nase) began in the early 1970's in the assessment of antibody binding to distinct antigenic determinants on the molecule (222). However, it was not until about five years later that the implications of these results were analyzed in the light of genetic control of the response to Nase at the determinant level (223,224,225).

Lozner et al. (86) mapped the antibody response gene, *Ir-Nase*, to the same subregion of *H-2* which was found to regulate the response to an
IgG myeloma (226,227), to what became known as the I-B subregion. The original assay for detection of anti-Nase antibody involved the inhibition of nuclease enzymatic activity, imposing constraints in assaying total anti-Nase responses. When a subsequent careful analysis of the response was undertaken (223), a number of interesting aspects emerged: (1) repeated immunization with Nase of a low responder strain eventually produced levels of antibody matching, and even surpassing, the levels produced by a congenic high responder strain, indicating a complex control, (2) non-H-2 control similar to that observed in the HEL system was seen, where A background mice produce 10-fold higher levels of antibody compared to B10 background strains, and, (3) peptide fragments of nuclease were found to share some of the immunogenic properties of the native molecule while differing in others: CNBr fragment 99-149 was found to mimic the genetic pattern of responsiveness to Nase with the exception of one strain which was found to be non-responsive (C57BL/10); the anti-Nase antibody of this strain was found to react with peptide 1-126 instead. However, antibodies to fragments 1-126 and 99-149 were found to cross-react very poorly with the native nuclease indicating a large role for stringent conformational dependence in the antibody response. In a subsequent analysis of high and low responding strains on the same background, it was confirmed that Ir genes control the determinant specificity of the response to nuclease (224) whereas non-H-2 genes control the magnitude of the response (228). Backcross analysis of the magnitude of the response indicated that the control was segregating independently from $\mathcal{V}_H$ and $\mathcal{C}_H$ gene loci.
The T-proliferative response to staphylococcal nuclease was described (225,229): the genetic assignment to I-B was confirmed, however, evidence for the involvement of other loci was considered, as different patterns of cross-stimulation of proliferative populations were observed with the use of Nase peptides. The immunizing dose was found to be critical in the initial analyses (85,230), as an increasing dose altered the patterns of responsiveness with additional genes tentatively localized to the right of I-J. A dichotomy between T- and B-cell recognition was observed: the lack of cross-reactivity between anti-peptide and anti-native nuclease antibody was not seen at the level of T-cell recognition, as not only good proliferation was seen to all the peptides, but in fact some peptides stimulated a higher proliferative response than the native molecule.

Further studies mapped the antibody response of H-2^d mice to I-A and H-2^k and H-2^a mice to I-B (231). This exclusive or non-allelic type of response most clearly defined that determinants in a complex antigen induce independent immune responses and conversely, that each Ir gene confers its own fine specificity of control of the immune response. The proposal was put forward that associative recognition of the antigenic determinant and the Ir gene product by T-cells could explain specificity of the response and that different subregions controlling the specificity could all perform the same functions. Despite the lack of cell surface antigens encoded by I-B, the response was definitely mapped to I-B and found not to be a result of complementation between I-A and I-E subregion (232).
Nuclease was one of the first antigens where a response under Ir gene control was used in idiootypic analysis: mouse anti-nuclease antibodies, purified on peptide immunoadsorbents, were used to immunize rats to produce anti-idiotypic antisera whose activity was initially determined in the antibody-mediated nuclease inactivation assay (233). The genetic control of idiootype expression was then investigated, and at the time it was found to be independent of H-2 linked Ir genes for expression (85,234) but linked to the heavy chain allotype locus. A high degree of idiootypic sharing was found amongst the strains tested. In further studies, anti-Nase antibodies reactive to a molecule comprising residues 1-99 demonstrated the same pattern of idiootypic reactivity as the whole anti-Nase populations, whereas antibodies to 99-149 indicated a separate idiootypic pattern (235). On this basis, five nuclease idiootypes were established, which increased the knowledge of a possible variable region gene map using BALB/c, CB.20 and BAB.14 mice, the latter strain resulting from a recombinational event which aided in tentatively mapping the Nase-specific idiotypic markers (235,236).

III. The merits of Fd as an immunological probe

The above is a compilation of observations pertinent to the regulation of T- and B-cell specific responses to a variety of proteins and peptides. It leads to a number of observations encompassing criteria for selection of an antigenic probe with the goal of recognizing and characterizing the interacting elements in an immune response.
Even with synthetic copolymers, both random and ordered, a vast amount of immunochemical research has been invested in the demarcation of the immunogenic sequences on an antigen. With synthetic molecules, the matter involves theoretically, the simple approach of constructing copolymers of fewer amino acids than found in the original molecule and testing the capacity of antibodies to the original molecule, to bind the ordered peptide. A good example of this work was seen in the (T,G)-A—L study described above, where Tyr-Tyr-Glu-Glu was found to the major determinant (71). Problems arise when it turns out that random polymerization produces an unknown number of determinants which vary from batch to batch in the production of an antigen. This problem was witnessed in the GLT system (56).

Work with naturally occurring protein antigens has at least eliminated the major problems of antigenic variability seen with some synthetic molecules. However, a few other problems have taken their place, problems inherent both in the approach to the research and the physical characteristics of the antigen:

1. Most, but not all protein antigens, notably HEL and BSA, have an exquisite dependence on protein conformation: native and denatured molecules stimulate different, sometimes non-cross-reactive spectra of antibodies (15). For example, the rigid triple helix of collagen induces a largely T-independent response (104), whereas the denatured molecule (gelatin) is T-dependent with two proposed determinants: Pro-Gly and Gly-Pro (10).
2. Antigens such as HEL with discontinuous topographic determinants present a monumental task in assigning specific amino acids to these determinants (237,238).

3. Although peptides can be synthesized according to predicted determinant sequences of the native molecule, conformationally correct fragments comprise only a small percentage of the molecules synthesized, as noted by the fact that soluble preparations usually have several thousand-fold lower affinity for antibody than the native molecule (120,215,238).

4. Enzymatic or CNBr peptides generated in most systems (100,134,182,212) still yield relatively large molecules, the danger in this approach being that assumptions are usually made that these fragments are unideterminant, whereas in reality two (or more) determinants may exist on each peptide.

5. As a number of determinant locations are deduced from comparisons of biological variants of the prototype molecule, a controversial situation exists. The question that needs to be conclusively answered is: are determinants localized in sequences where amino acids differ from the homologous counterpart or are determinants conformationally dependent and present on both variant and homologous proteins, initiating autoimmunization? Proponents of both hypothesis exist: studies with HEL (176,177), cytochrome c (110,111), and insulin (142,155) support the notion that immunogenicity exists to sequences not shared with the autologous molecule, whereas other studies with insulin (144), myoglobin
(195,196), albumin (96), and cytochrome c (126) put forward a case for autosensitization and conformational determinants irrespective of cross-reactions with self counterparts.

6. With the advent of hybridoma technology, determinants not previously uncovered through extensive immunochemical studies are found to react with considerable proportions of monoclonal antibodies. As was seen above, none of twelve monoclonals to myoglobin bound either of the two antigenic regions (residues 1-55 and 132-153) which had previously been implicated in binding 70-90% of serum antibody (216).

7. The general acceptance that T-cells recognize sequential determinants and B-cells recognize either the same determinants (20,84,213) or partially or totally different topographical ones (80,85,102,135,152,230), complicates both the assignment of immunoregulatory sequences and the building of models. Usually, models depict both T- and B-cells recognizing the same or similar looking antigenic forms in both idiotypic and antigenic-bridging networks.

8. In some cases, the native antigens that are used have non-immunologically related detrimental effects on the immunized animal (e.g. glucagon). At other times autoimmunity can be induced by immunization with biological variants (e.g. insulin)

The above cited difficulties in most cases are not insurmountable, but require judicious interpretation of the results. With ferredoxin, the majority of these problems are satisfactorily eliminated:
a. due to its small size, both native and denatured forms of Fd impart identical antibody responses and genetic control.

b. as Fd is a non-mammalian protein, it has no autologous counterparts, thus eliminating autosensitization as well as determinant masking induced by self tolerance. Through sequence homology studies (239,240), at most 20% identity with cytochrome c (from Desulfovibrio vulgaris) has been found, confirming a previously established cross-reactivity at the C-terminus, however, this is not thought to play any demonstrable role in autosensitization.

c. since linear determinants (not discontinuous or topographically dependent) are recognized, within the present limits of analysis, T- and B-cell repertoires can be easily compared and in fact conclusively determined in future studies through T-cell cloning and T-hybridoma technology;

d. monoclonal antibodies produced to date in this laboratory all have either N-or C-determinant reactivity, hopefully indicating that only these specificities will be uncovered and Fd will continue to be strictly a two determinant system at the B-cell level.

e. since only two determinants exist (at the B-cell level), selected fragments can be obtained which carry only one of the antigenic determinants.

f. Fd has not been found to cause any detrimental side effects both in vivo and in vitro.

As a result of the studies undertaken for this thesis, as well as ongoing research in this laboratory on the idiotypic aspects of the response
(241,242,243,244,245,246), Fd is proving again to be a very profitable immunoregulatory probe. The following are the major contributions to the Fd system as described in greater detail in further chapters of this thesis:

1. Genetic analysis of the anti-Fd response has demonstrated that phenotypically different responses both at the antibody and T-proliferative levels could be ascribed to H-2 linked genes to the exclusion of non-MHC genes. Insofar as the antibody response is concerned, this genetic control maps to the K/I-A regions, with the k haplotype demonstrating high antibody levels to Fd, the b and s alleles of K/I-A representing intermediate response levels, while H-2d mice show neither an antibody nor a proliferative response to the native molecule.

2. Through selective enzymatic digestion, undeterminant fragments of Fd were generated. These were employed in the assessment of determinant specificity of anti-Fd antibody. A genetically determined ratio of "N" – versus "C"-reactive antibody was mapped to the same K/I-A region, with the indication that intermediate antibody producers demonstrate roughly equivalent reactivity to the two determinants, while the high responders reflect a net increase in only "C"-specific antibody. The doubly digested peptide ("M", devoid of functional determinants) was used to confirm the two determinant status: "M" binds no anti-Fd antibody.

3. These three fragments of Fd, "N", "C" and "M" were used as immunogens with very exciting results: whereas "M" did not induce an antibody response, and "N" was a very poor immunogen, "C" has
proven to be significantly more immunogenic than the native molecule, to the point of inducing an antibody response in H-2\(^d\) (non-responder) mice. This is felt to be a critical finding in the implication of the N-terminal tripeptide in suppressor functions.

4. The comparison of antibody and T-proliferative responses has provided a basis for the re-evaluation of the proliferative assay. Evidence provided by studies at the unideterminant level demonstrate that caution must be exercised in the interpretation of proliferation data.

5. Finally, preliminary statements concerning the nature of the non-responsiveness have been forwarded with the use of adoptively transferred immune T-cells.
Chapter 2. Materials and Methods

I. Experimental animals

B10.S, B10.S(9R) and B10.HTT breeding pairs were obtained from Dr. D.C. Shreffler, Department of Human Genetics, University of Michigan, Ann Arbor, Michigan. Female NZB mice were a generous gift from Dr. A. Steinberg, Arthritis and Rheumatism, Branch, National Institute of Health, Bethesda, Maryland. B10.A(2R), B10.A(3R) and B10.A(4R) mice were bred by Dr. H.-S. Teh, U.B.C. AKR/J, A.SW, C57BL/10 Sn, B10.A/SgSn, B10.D2/0Sn, C3H/HeDiSn, DBA/2J, CE/J, C58/J, CBA/J, ST/J, RF/J, SJL/J, BALB/c, SM/J and B6D2F1/J mice were obtained either as adult animals or as sibling mating pairs and bred in our animal facility. (B10.BR x B10.HTT)F1, (B10.BR x B10.D2)F1, (B10.BR x B10.S)F1, (B10.BR x B10.S)F1, (B10.S x B10.D2)F1 and (CBA x DBA/2)F1 mice were bred in our animal facility. Whenever possible, female mice were used at two to six months of age. The relevant genetic information is detailed in Appendix I.

II. Preparation of antigens

A. Ferredoxin

Ferredoxin was isolated and purified from Clostridium pasteurianum (ATCC-2 6013) using a modification of the methods described originally by Mortenson (247) and Tanaka et al (248). The protocols for
growing the bacteria and purifying the extracts are described in extensive
detail in Appendix II. Ferredoxin was also purchased from Sigma (F7629).

B. TCA-Fd

Iron-sulfide free ferredoxin (TCA-Fd) was prepared according to
the method of Tanaka et al (248).

C. Enzymatic fragments of TCA-Fd

1. The "N" molecule. TCA-Fd was dialyzed (Spectraphor 6, 2000
M.W. exclusion) into 0.025 M Tris buffer with 0.5 M NaCl and 10 mM
2-mercaptoethanol at pH 7.5, and the enzyme, carboxypeptidase A (CPA)
[Worthington, COAPMS], whose activity was determined by the method of Folk
et al (249) described in the Worthington Biochemical Corporation, "Enzymes
and Related Biochemicals" catalogue, 1978, p. 27, was dissolved in 10%
LiCl and added in 0.01 ml aliquots at 8-hour intervals to a final 2.5% w/w
enzyme to Fd concentration ratio. The reaction was carried out under N₂
in a tightly screwed-cap vial at room temperature for 4 days with gentle
stirring. The extent of the digest was followed by ELISA using standard
serum pools (of known "N" versus "C" reactivity) and monoclonal antibodies
of defined anti-Fd specificity. It was found that at times this procedure
was inadequate in achieving complete digestion of the terminal two amino
acid residues. According to Green and Stahman (250) and Titani et al
(251), the cleavage of glutamic acid residues by CPA is extremely slow at
pH 7.5 and may be improved by lowering the pH down to 5.0. This however,
neccessitates increasing the enzyme concentration to 10% w/w due to CPA
instability at the lower pH. A compromise was achieved by lowering the pH
of the buffer with a small amount of 1.0 M HCl for the first day and then
readjusting it to pH 7.5 with 1.0 M NaOH after 24 hr, thus initiating some of the terminal glutamic acid cleavage. The enzyme was added 1% w/w every 6 hr for the first 24 hr, and then every 12 hr for a total of 10% w/w in 4 days. This procedure removes the gln-glu-COOH residues at the COOH terminus of the molecule and effectively eliminates the immunological properties of one of the two antigenic determinants in the Fd molecule. CPA digestion is naturally terminated at this point because of a proline residue at the 52 position of the Fd molecule.

2. **The "C" molecule.** TCA-Fd was dialyzed (Spectraphor 6, 2000 M.W. exclusion) into 0.046 M Tris buffer with 0.0115 M CaCl₂ and 10 mM 2-mercaptoethanol at pH 8.1. The enzyme trypsin (TR) (Worthington TRTPCK) was added in three 0.01 ml aliquots at hourly intervals to a final concentration of 2.5% w/w of enzyme/Fd. The reaction was carried out to completion at room temperature under N₂, stirring, overnight in an air-tight vial. The completeness of the digest was assessed by ELISA using standard serum pools and monoclonal antibodies. Because the only trypsin sensitive region in Fd occurs at the lys-ile bond at positions 3 and 4 of the Fd molecule, this digestion procedure effectively eliminates the N-determinant while leaving the remainder of the molecule (52 residues) intact.

3. **The "M" molecule.** Carboxypeptidase A digestion described above was followed by dialysis of the completed digest into the appropriate buffer for trypsin digestion, which was carried out to completion as described above, with a final TR concentration of 10% w/w. Total lack of reactivity with any anti-Fd reagent in the ELISA was the
measure of completeness of the reaction. The "M" fragment thus constitutes the Fd molecule devoid of 3 amino acids at the NH$_2$-terminus and 2 amino acids at the COOH-terminus.

As all the above fragments exhibit a degree of aggregation, for most experiments the fragments were not purified from the enzyme containing digest as this would have made quantitation inaccurate. Where such purification was necessary, methods described in Appendix III were used. In most experiments, animals were immunized with the crude enzyme digests. It was determined that trypsin was totally non-immunogenic in both rabbits and mice, and mice were not rendered CPA-immune with the low levels of immunizing CPA used in the digests, although an anti-CPA response was obtained in rabbits using a higher immunizing dose. At any rate, fragment immune mice were always tested for anti-Fd reactivity (both antibody and T-proliferative responses) so that the enzymatic contamination was never an issue in terms of the response being measured. As digestion with either enzyme yields at most a tripeptide contaminant which is dialyzable, further complications with the fragments were not anticipated, since the presence of undigested Fd can be ruled out by the ultrasensitive ELISA analysis using monoclonal antibodies to monitor for the presence of either N- or C- determinants in the digests.

III. Sera

Mice were immunized subcutaneously in both flanks with a total of 0.2 ml containing 50 µg Fd or "N", "C" and "M" in saline emulsified 1:1 in CFA, H37Ra (Difco Laboratories, Detroit, Michigan). Control mice received either 50 µg KLH (Calbiochem #374805) or just saline emulsified
in CFA. Primary sera were obtained on days 9, 14, 18, 21 and 35 after priming, depending on the experiment. Etherized mice were bled from the tail vein. Four or more weeks after the primary immunization the mice were boosted as in the primary stimulus. Sera were obtained seven days later either by tail vein bleeding or after the mice were sacrificed. Sera were stored individually at -20°C. The ELISA described below was used in determining the titre and specificity of the sera.

IV. Monoclonal antibodies

The monoclonal antibodies used in the specificity testing of the enzymatic digests of Fd were produced in this laboratory by Michael Weaver and Doug Demetrick, with use of current hybridoma technology and described in detail elsewhere (241). Anti-Fd monoclonal antibody #6-90 was shown to be specific for the N-determinant while #17-3 and #DFd-2E were shown to be specific for the C-determinant.

V. Antibody assays

A. Standard ELISA

The enzyme-linked immunosorbent assay described originally by Voller et al. (252), was used for detecting both native Fd and determinant-specific antibody. Polystyrene ELISA plates (Dynatech M129A) were coated with 0.1 ml of a 1-5 μg/ml solution of the desired antigen in coating buffer (carbonate-bicarbonate pH 9.6) and were incubated overnight at 37°C. For screening responding versus non-responding animals, sera were tested at a 1:100 dilution in PBS-Tween buffer in triplicate or quadruplicate. The developing antiserum was alkaline phosphatase labelled rabbit anti-mouse Ig (RaMIg) diluted in PBS-Tween
and prepared as described previously (253). The enzyme substrate, p-nitrophenyl phosphate (Sigma 104-105), was dissolved in diethanolamine buffer pH 9.8 and the reaction was allowed to proceed in most experiments for 30 min, at which time the absorbance at 405 nm was read on the Titertek Multiscan (Flow) spectrophotometer. In the earlier days of this research, the individual wells were diluted 1:4 with distilled H₂O and the absorbance was read on a Beckman DBG spectrophotometer at 405 nm.

The ELISA was also used in the analysis of hybridoma culture supernatants whether for the detection of antibody production of B-cell fusion products or putative idiotypic helper or suppressor factors secreted by T-cell fusion products; the latter application is discussed in Appendix VIII. Appendix IV covers the use of the ELISA in determining antibody production in primary and secondary in vitro cultures of splenic B-cells responding to Fd. The ELISA is also used extensively in the purification of Fd as described in Appendix II.

B. Blocking ELISA

In genetic mapping experiments, six individual sera from each strain of mice were titrated using the standard ELISA. The plates were coated overnight with 0.1 ml of Fd at 4.0 µg/ml. The tests were done in quadruplicate. The dilution of each serum which gave an absorbance reading at 405 nm of 0.5 ± 0.1 after 60 min incubation of plates at room temperature was chosen as the dilution in which the blocking assay was carried out. In a previous study (253), it was found that A₄₀₅ₙₐₖ intensity could be directly correlated with the amount of specific antibody found, so the use of this method to establish baseline reactivity
of a given antiserum is valid, in terms of the assumption that, under these conditions, essentially equivalent amounts of antibody are titrated in each instance. In high responder animals this dilution was on the average 1:1200 and in intermediate responders about 1:300. The low responder strain, B10.HTT was tested at 1:100 and the ELISA was incubated for 18-24 hours, since so little specific antibody was present in the serum of these animals.

The blocking test was carried out by overnight incubation of the Fd-immune sera with different concentrations of the enzyme digested Fd fragments (N– and C-determinant-bearing molecules): 0.5, 1.0 and 2.0 μg/ml final concentrations were used with all sera; all results were in the linear range. Blocking assays were carried out at three concentrations as a measure that all sera tested were titrating in a manner similar to those sera which were originally titrated over a broader range of "hapten" concentrations, at which time soluble Fd was run as a positive control for 100% inhibition of the ELISA. The relative N– vs C-determinant reactivity was calculated as follows:

\[
\% \text{ N-determinant reactivity} = \frac{n}{n+c} \times 100\%,
\]

where

\[
n = \text{amount of inhibition of the response measured on the native Fd coated plate by the N-determinant bearing molecule, and}
\]

\[
c = \text{amount of inhibition of the response measured on the native Fd-coated plate by the C-determinant-bearing molecule.}
\]
Values were calculated for each of the three dilutions of unideterminant fragments used in the blocking and averaged since the N:C ratio of reactivity was constant at the three concentrations of blocking antigens. The values that are described are the average for six sera ± S.E.

C. ELISA-RIA

A radioimmunoassay was used in conjunction with the ELISA in order to quantitate the amount of Fd-specific antibody in the serum of immune animals. Anti-Fd antibody was purified using Fd coupled to thiopropyl-Sepharose B (Pharmacia, Uppsala, Sweden) immunoadsorbent with procedures described elsewhere (241). Antibody from Fd-immune B10.BR mice was eluted with 0.1 M glycine buffer pH 2.3 and neutralized immediately with 2.0 M Tris buffer pH 7.2 and quantitated by ELISA. It was subsequently labelled with $^{125}$I by the method of Greenwood et al (254) with a resulting specific activity of $2.27 \times 10^7$ cpm/mg Ig. The standard ELISA was set up with this radiolabelled antibody, the colour development after 30 min was recorded; the wells were then cut out and counted in a gamma counter (Beckman Biogamma). The amount of specific Ig adhering to the plate was calculated from its specific activity and correlated to the color development. Despite the recommendation by Butler and co-workers (255) that ELISA results be expressed in absorbance units and not µg/ml of antibody due to the influence of affinity on colour development, it was felt that such quantification was justified as comparisons were made within a single system.
VI. Proliferation assays

A modification of the method described by Lee et al. (256) was chosen as the technique most applicable to single animal studies of the T-cell proliferative response to Fd. Mice primed and boosted with 50 μg Fd (or one of the enzymatic fragments) in 0.2 ml CFA subcutaneously in both flanks. Seven days after the secondary immunization, the draining inguinal and lumbar (periaortic) lymph nodes were excised immediately after the animal was sacrificed by cervical dislocation, teased apart to a single cell suspension in PBS supplemented with 5% heat inactivated foetal calf serum (FCS) (Gibco Laboratories #200-6140), washed twice in PBS + 5% FCS, resuspended in RPMI 1640 (Flow Laboratories 10-601-24) containing 3.5 g per l NaHCO₃, (± 10 mM HEPES), and 50 ng per l gentamycin (Sigma G3632) or penicillin-streptomycin (Gibco Laboratories #200-6140), and counted using trypan blue exclusion. 1.6 x 10⁶ cells in 150 μl medium were dispensed per well into 96-well Linbro microtitre plates (IS-FB-96TC); 0.05 ml of 50% human serum which was heat inactivated (30 min, 56°C) and stored at -70°C in aliquots, was added to reach a final concentration of 10% (v/v). Fd (or the enzyme digested fragments) was added to a final concentration of 50 μg/ml. The animals were tested individually with and without Fd; each test was run at least in triplicate. A PPD (Connaught Laboratories) (or KLH, where applicable) control was run when sufficient cells were available. Incubation was at 37°C in a humidifed atmosphere and 10% CO₂. After 4 days, the cell cultures were pulsed for 22-24 hours with 1μCi ³H-met-Thymidine (NEN; NET-027A) in 50 μl medium and harvested with a MASH harvester, onto
fiber filters (Whatman 934 AH), dried and counted in 2 ml toluene omnifluor (Syndell, Vancouver) scintillation fluid in a Unilux II scintillation counter (Nuclear Chicago). The stimulation index for each animal was computed as follows:

\[
\frac{\text{Average cpm with Fd}}{\text{Average cpm without Fd}}
\]

In certain studies both stimulation indices and net counts were employed in the analysis. There are several factors which are important in optimalizing this assay and they are discussed in Appendix V.
Chapter 3. Results: Genetics of the Response to Native Fd

Preliminary experiments addressed the question as to whether mouse strains demonstrated genetically based differences in their immune response to the native molecule as demonstrated by both cellular and humoral tests of immunity. Mice differing at the MHC as well as in non-MHC genes were immunized subcutaneously in the flanks with 50 μg of Fd in CFA (the optimum immunizing dose, see Figure 1); they were rested a minimum of 30 days and then boosted using the same protocol. Seven days after the secondary challenge, the mice were sacrificed by cervical dislocation, the blood was collected immediately from the thoracic cavity after heart puncture; the serum was frozen, and saved for ELISA testing. The draining lymph nodes were excised and used in vitro, in proliferative assays.

I. Choice of proliferation assay

At the time that this research was initiated, only the PETLES assay devised by Schwartz et al. (116) was available as a specific and reliable determination of T-cell responses to soluble proteins and synthetic antigens. In a preliminary study, the PPD response was assessed using PETLES as well as unfractionated and nylon wool purified (257) lymph node and spleen cells. The indications were (data not shown), that in our hands, lymph node cells, whether enriched by nylon wool or not, demonstrated the most significant T-cell proliferation, whereas the other sources of T-cells seemed rather inadequate. A significant problem with
Figure 1. Fd dose response curve. B10.BR mice were immunized in complete Freund’s Adjuvant subcutaneously with 0 to 100 μg Fd, rested 30 days, re-immunized with identical doses of antigen in 50% CFA and bled from the tail vein. The ELISA was carried out with plates coated with Fd at 1 μg/ml; sera were tested at a 1:250 dilution; the reaction was recorded at 30 min.
this assay is that for the best response using nylon wool purified lymph node cells, only pools of cells from many animals can be used, and assessment of the T-proliferative potential of individual mice could not be made. These observations were corroborated by Corradin et al. (117), who also described the superiority of the proliferative response of draining lymph node.

The Corradin assay was used for six months. At that time, Lee and co-workers (256) communicated the development of a much improved assay that was subsequently used with a few modifications. In Table II, a comparison between the Corradin and Lee assays is made, and a few general statements may be made about the average results: although high net counts are observed with the Corradin assay (Fd: 14,755 ± 1,289, non-immune control: 4,895 ± 1,795), these are accompanied by high background counts [Fd: 5,678 ± 631, control: 6,277 ± 1,117], resulting in rather poor stimulation indices [Fd: 3.98 ± 0.49, control: 1.75 ± 0.15]. In contrast, although twice as many cells were employed in the Lee assay, extremely low background counts [Fd 229 ± 23, control: 1645 ± 1082] are observed, reflecting the much superior stimulation indices [Fd 39.47 ± 14.28, CFA: 3.40 ± 1.60], despite the relatively low net counts [Fd: 10,857 ± 5,535, control: 1,253 ± 1,007]. Thus, a relatively inexpensive [RPMI 1640 is much cheaper than the EHAA medium described by Click et al. (258), as is the human serum obtained at no cost from the Red Cross as opposed to FCS] assay became available for the assessment of T-cell immunity in single animal studies of the genetics of the immune response.
Table II - Comparison of the T-cell proliferation assays described by Corradin et al (117) and Lee et al (256)

<table>
<thead>
<tr>
<th>Immunizing antigen&lt;sup&gt;c&lt;/sup&gt;</th>
<th>CULTURE WITH - Fd + Fd net</th>
<th>S.I.&lt;sup&gt;d&lt;/sup&gt;</th>
<th>CULTURE WITH - Fd + Fd net</th>
<th>S.I.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
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</tr>
<tr>
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<td>15,007</td>
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<td>8,300</td>
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<td>5,820</td>
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</table>

a) A modification of the assay described by Corradin et al was used: 4 x 10<sup>5</sup> unseparated inguinal lymph node cells were used per well, 20 μg/ml NEM-Fd, EHAA medium supplemented with 5% foetal calf serum, 5 day culture, 24 hour <sup>3</sup>H-Thy pulse.

b) A modification of the assay described by Lee et al was used: 8 x 10<sup>5</sup> unseparated inguinal lymph node cells were used per well, 20 μg/ml NEM-Fd, RPMI 1640 supplemented with 10% human serum, 5 day culture, 24 hour <sup>3</sup>H-Thy pulse.

c) CBA/J mice were primed and boosted with 50 μg NEM-Fd or saline in CFA subcutaneously in the flank. Although mice in both assays were immunized at the same time, the same mice were not used in both assays.

d) S.I. = Stimulation index = \( \frac{\text{net cpm} + Fd}{\text{net cpm} - Fd} \)
II. **Proliferation assay: cell number and antigen dose**

In order to optimize the proliferative response, cell number and antigen concentration were titrated; the results are shown in Table III. The PPD response is used as a positive control, while KLH serves as the negative one. Although in this particular experiment the best response was obtained with $2 \times 10^6$ cells per well, this represented a pooled cell population from a number of animals whereas in single animal studies, in which a maximum of $1 \times 10^7$ cells from both the inguinal and periaortic lymph nodes can be obtained, it was decided that the routine screening of proliferative responses would be at a lower number ($1.0 - 1.6 \times 10^6$ cells per well). Similarly, in this particular experiment, the optimal antigen dose was 100 µg per ml for Fd (and 50 µg per ml for PPD), however, due to the cost of the antigen, this dose was lowered to 50 µg/ml.

III. **The proliferative response: linkage to the H-2**

Figure 2 demonstrates the proliferative responses of a number of strains of mice: $H^{-2^K}$ (B10.BR and CBA) and $H^{-2^S}$ (B10.S and SJL) mice were found to be proliferators, whereas $H^{-2^b}$ (C57BL/10) and $H^{-2^d}$ (B10.D2, BALB/c, DBA/2 and NZB) mice showed no proliferation in response to Fd. Subsequent studies, described later, show A.SW mice which are $H^{-2^S}$, to have a stimulation index of $32.25 \pm 6.29$ (control $1.84 \pm 0.33$) placing them in the range of other responder strains. SJL mice have a substantially higher response as compared to other $H^{-2^S}$ mice. Because of uncertainties regarding the validity of lymphocyte stimulation as an absolute measure of immune responsiveness, further genetic mapping was carried out using antibody synthesis to Fd.
Table III - Titration of cell number and antigen concentration in the proliferation assay

<table>
<thead>
<tr>
<th>Immunizing antigen</th>
<th>Cell number per well (x 10^5)</th>
<th>antigen in culture</th>
<th>antigen concentration (µg/ml)</th>
<th>net cpm</th>
<th>stimulation index</th>
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<td>100.0</td>
<td>-14</td>
<td>0.95</td>
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a) A.SW mice were used

b) B10.BR mice were used
Figure 2. T-cell proliferation assay of four major haplotypes. The draining lymph node cells of mice which had received 7 days previously a secondary immunization of 50 µg Fd in CFA subcutaneously in both flanks were suspended at 1.6 x 10^6 cells/well in RPMI 1640 + 3.5 g/l NaHCO₃ with 10% heat inactivated human serum and Fd at a final concentration of 50 µg/ml. Incubation was for 5 days at 37°C, 6% CO₂ with a 22- to 24- hr ³H-met-thymidine pulse. The stimulation index is the ratio of counts with and without Fd ± S.E. Responses of immune animals (upper bar graph) are compared to non-immune animals (lower bar graph).
IV. Choice of antigen

A number of modified ferredoxins (O-Fd, Meth-O-Fd, NEM-Fd, TCA-Fd, DNP-O-Fd and CM-Fd) were used in a previous study (12) to assess cross-reactivity of these antigens with rabbit anti-OFd antibody and in murine lymphoproliferative responses. Various levels of complement fixation were obtained with the use of these ferredoxin preparations and the rabbit antiserum, and a strong response was observed with native Fd, O-Fd, DNP-O-Fd and TCA-Fd, an intermediate to poor reactivity with NEM-Fd and CM-Fd and no reactivity with meth-O-Fd. When these preparations were used to stimulate both DBA/2 and B6D2F1 spleen cells, it was observed that the best proliferation was obtained with NEM-Fd. For this reason, initially all mice were immunized with NEM-Fd.

When the ELISA was being set up, murine anti-NEM-Fd were tested on the immunizing antigen, native Fd and CM-Fd (forms which previously gave variable complement fixation results). The results of such an assay with SJL serum are seen in Figure 3 and indicate that although native Fd is again superior in this assay, good reactivity is seen with all three preparations.

The validity of the preference for the use of NEM-Fd can be also challenged on the basis that the previously performed lymphoproliferative assays was carried out with non-responder animals, and with spleen cells (which give notoriously poor antigen specific proliferation) and at very low antigen concentration (16 µg/ml). Therefore, although all initial genetic studies were carried out with mice immunized with NEM-Fd, all subsequent studies employed native Fd.
Figure 3. Comparison on ELISA of Fd, CM-Fd and NEM-Fd. NEM-Fd immune and non-immune pooled SJL sera were used at 1:200 on ELISA plates coated with Fd, CM-Fd and NEM-Fd at 1.0 μg/ml. The 30 min reading is shown.
These data point to one very important observation: anti-Fd antibody binds both native and denatured forms of the antigen indicating that in this system and within the limitations of these assays, the response to Fd is not dependent on the conformation of the molecule.

V. Quantification of anti-Fd antibody

Actual concentrations of Fd-specific serum antibody were determined using a combined RIA-ELISA, where reactivity of $^{125}$I-labelled serum antibody to Fd bound to a polystyrene plate was correlated to the enzymatic colour development by alkaline phosphatase-linked rabbit anti-mouse immunoglobulin reacting with the specific antibody. The relationship of colour development at $A_{405\text{nm}}$ to the amount of specific antibody (a function of counts bound to the plate and the specific activity of the protein) is shown in Figure 4. With the use of this dose-response curve, it was possible to calculate from ELISA's the amount of specific anti-Fd antibody in the serum of mice. In subsequent experiments, both µg/ml antibody and $A_{405\text{nm}}$ were used to define antibody levels in serum samples.

VI. Linkage of antibody response to H-2

The serum antibody of the mice previously tested in the proliferation assay was measured by ELISA. Figure 5 summarizes the data obtained. A clear three level distinction can be made: high responder status may be assigned to H-2$^k$ strains, intermediate status is linked to H-2$^s$ and H-2$^b$ and all H-2$^d$ mice are non-responders.

In the comparison between the two methods of assessment of anti-Fd immunity, proliferation and antibody production, correlation is observed.
Figure 4. ELISA-radioimmunoassay calibration curve. Fd (1µg/ml)–coated ELISA plates were treated with affinity purified B10.BR anti-Fd antibody labeled with $^{125}$I. The enzymatic color development with alkaline phosphatase-linked rabbit anti-mouse Ig was measured for absorbance at 405 nm and compared to the counts per well. The results were related to ng antibody from the specific activity of $2.27 \times 10^7$ cpm/mg protein. The dotted line represents the non-linear part of the graph.
Figure 5. Antibody response of four major haplotypes. Mice were primed and boosted with 50 μg Fd in CFA subcutaneously in both flanks. Seven days after the secondary immunization, the ELISA was performed on Fd coated plates at a 1:100 dilution of antiserum. The absorbance at 405 nm was related to ng antibody by using figure 4. Each result represents 12 mice on the average ± S.E. Non-immune sera were between 0 and 1 μg/ml serum and are not shown.
<table>
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<th>HAPLOTYPE</th>
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<td>k</td>
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<tr>
<td>C3H</td>
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<td>CBA</td>
<td>k</td>
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<td>s</td>
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<tr>
<td>C57BL/10</td>
<td>b</td>
</tr>
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<td>BIO.D2</td>
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</tr>
<tr>
<td>BALB/c</td>
<td>d</td>
</tr>
<tr>
<td>DBA/2</td>
<td>d</td>
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<tr>
<td>NZB</td>
<td>d</td>
</tr>
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</table>

microgram antibody per milliliter serum
with $H-2^k$ and $H-2^d$ strains, high and non-responders in both systems.

Problems arise in the correlation between the two assays with $H-2^b$ and $H-2^s$ strains. First, $H-2^b$ mice make an intermediate level of antibody yet demonstrate no proliferation and secondly, some $H-2^s$ mice which make equivalent levels of antibody, show proliferation equalling or even surpassing (SJL) the proliferative response of high antibody responders. This, as will be seen through the remainder of this thesis, is the first example of dissociation between the two parameters of immune responsiveness.

VII. **Linkage to heavy chain allotype genes**

Three $H-2^k$ strains were tested in Figure 5. All were demonstrably high responders with significantly higher responses than in $H-2^b,s$ and $d$ strains, yet no significant difference was demonstrated between them. As B10.BR mice bear the IgCH* allotype and both C3H and CBA are IgCH*, a wider spectrum of allotypically different but H-2 congenic mice were tested in order to assess whether there are any allotype-linked, non-H-2 effects on the magnitude of the antibody response. Figure 6 shows the results obtained. Although a variation is seen from strain to strain, there is no correlation to either the heavy nor the light chain allotypes. If there is any non-H-2 control over the magnitude of the anti-Fd response, there is no available genetic basis of analysis to determine this effect.

VIII. **Mapping of the response within the H-2**

So far, it is clear that non-MHC genes cannot be strictly implicated in the control of the immune response to Fd and that $H-2^k$ control defines $H-2^d$ as high responders, $H-2^b,s$ as intermediate and
Figure 6. Antibody response of H-2\(^k\) mice with various allotypes. Mice were immunized and tested according to the protocol described in figure 5. Results are shown for the absorbance value at 405 nm obtained after 30 min ± S.E.
H-2\textsuperscript{d} mice as non-responders. Further localization within the MHC of the genes responsible for the regulation of the antibody response was undertaken with the use of the recombinant strains available on the B10 background i.e. the C57BL/10 strain provides all non-H-2 genes and through one or more recombination events, strains are generated which carry a variety of H-2 alleles including intra-H-2 recombinations.

The ELISA results of the antibody responses of various recombinant strains are shown in Figure 7. The B10.A(2R) and B10.A(4R) strains make as much antibody as B10.BR's and more than all other strains tested. Similarly, B10.A(3R), B10.A(5R) and B10.S(9R) recombinants are classified as intermediate responders with responses not significantly different from the congenic intermediate responders B10.S and C57BL/10 (P \leq 0.25). Based on these results, mapping of the response gene(s) was accomplished:

B10.A(4R) mice critically define the gene to the K/I-A as they are high responders to Fd characterized by the k allele and not intermediate responders due to the b allele at I-B, I-J, I-E and I-C. This result is supported by the response of B10.A(2R) mice which also bear the I-A\textsuperscript{k} high responder gene (as well as I-E\textsuperscript{k}). In the converse situation, B10.A(5R) mice which carry the k allele only in I-J and I-E do not mount a high response characteristic of the H-2\textsuperscript{k} haplotype but show intermediate responder status of the H-2\textsuperscript{b} haplotype again supporting the localization of the response to K/I-A/I-B. B10.A(3R) mice support this assignment. A similar situation is seen with the B10.S(9R) recombinant strain, which like B10.A(5R) mice bears I-J\textsuperscript{k} and I-E\textsuperscript{k}, yet demonstrates an
Figure 7. Antibody responses of recombinant mice. The tests were performed under conditions described in figure 5. Responses of B10.BR, B10.S, C57BL/10, and B10.D2 mice are shown for comparison. I region alleles of I-A, I-B, I-J, I-E and I-C are shown. Comparison of both B10.A(3R) and B10.A(5R) responses to that of C57BL/10 mice using the Student's t-test results in a p value < .25, indicating no significant difference, while the comparison to the B10.BR, B10.A (2R) and B10.A(4R) yielded values of p < .001, p < .4 and p < .001 respectively, indicating a significant difference from the response of C57BL/10 mice. In the comparison of B10.S and B10.HTT responses, a p value of .015 was obtained indicating a significant difference.
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<tr>
<td>C57BL/10</td>
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<tr>
<td>BIO.A(3R)</td>
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<tr>
<td>BIO.A(5R)</td>
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<td>b k k d</td>
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<tr>
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<td>s</td>
<td>s s s s</td>
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<tr>
<td>BIO.S(9R)</td>
<td>t4</td>
<td>s s k k d</td>
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<tr>
<td>BIO.HTT</td>
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<tr>
<td>BIO.D2</td>
<td>d</td>
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intermediate responder status identical to the B10.S strain, again localizing the response to K/I-A/I-B.

The only anomaly in this genetic analysis rests with the B10.HTT strain, which by the observations presented above would be expected to show an intermediate response as it carries the $I-A^s/I-B^s$ genes determining the intermediate status. However, B10.HTT make significantly ($p < 0.015$) less antibody than the B10.S and B10.S(9R) strains, with only a few mice making any measurable antibody; henceforth, this strain is denoted as a "low responder". The reasons for this are not understood, yet clues to this B10.HTT anomaly will appear in later experiments. For example, when the proliferative capacity of the two strains was compared in Figure 8, it could be seen that not only did B10.HTT produce inexplicably low levels of anti-Fd antibody but also it was unable to mount a reasonable proliferative response as compared to B10.S. Studies with $F_1$ mice indicate that the lack of proliferative capacity of the B10.HTT strain is not a dominant characteristic as (B10.BR x B10.HTT)$F_1$ mice proliferate to the same extent as the (B10.BR x B10.S)$F_1$'s. The suggestion put forward is that the lesion of the B10.HTT response is in all probability not a result of suppression, but rather a gene defect or mutation in the control of antibody production.

IX. Conclusion

In summary, the murine response to Fd is dose dependent, and antigenic form (native versus denatured) independent, both at the antibody and proliferative levels. The Lee et al (256) assay for T-cell proliferation was chosen as the most satisfactory one for single animal
Figure 8. Comparison of the proliferative responses of B10.S and B10.HTT mice. The proliferative assay was carried out as described in figure 2.
studies in the response to Fd. It was determined that H-2^d strains do not induce cells which can proliferate in vitro to Fd, H-2^b mice are also weak in this aspect while both H-2^s and H-2^k mice demonstrate very marked Fd-specific proliferation. The ELISA which can be quantitated to yield results in terms of µg/ml of specific antibody (although presentation of raw data i.e. A^405nm is perfectly acceptable) has permitted the assessment of the magnitudes of the antibody responses. H-2^k mice are high responders, H-2^b and H-2^s are intermediate antibody producers and all H-2^d mice make no anti-Fd antibody. Two major conclusions may be drawn: 1) non-H-2 genes do not play a major role in either T-cell proliferation or antibody production and 2) there is a lack of correlation between antibody synthesis and lymphocyte proliferation both quantitatively (magnitude of either response) and qualitatively (absence of proliferation does not preclude an antibody response as seen in C57BL/10). This H-2 linked antibody response was found to map to K/I-A with exception of the B10.HTT strain. This non-conforming strain demonstrates low antibody production and no proliferative response. This is thought to be a result of a genetic lesion either due to mutation or an uncharacterized recombination. It is believed that this low responsiveness is not due to active suppression, as at both the proliferative and antibody levels, the (B10.BR x B10.HTT)^F1 behaves normally, i.e. it proliferates as well as B10.BR and makes a gene dose dependent level of antibody. It may be pointed out at this time that whereas the antibody response is a gene dose dependent phenomenon, i.e. (R X NR)^F1 make intermediate levels of antibody, the proliferative
response is an all- or none-effect i.e. (R x NR)F₁ proliferate to the same extent as the responder. This case is very well illustrated with (B10.BR x B10.HTT)F₁, Figures 8 and 18.
Chapter 4. Results: Genetics of the response at the determinant level

In order to study further the immune response to Fd, it was necessary to establish the determinant specificity of the anti-Fd antibody. Previous results (3,4) strongly indicated that Fd, the sequence of which is shown in Figure 9, possesses two major antigenic determinants, one at the amino- and the other at the carboxy-terminal of the molecule. The amino-terminal heptapeptide is subsequently referred to as the N-determinant and the carboxy-terminal pentapeptide as the C-determinant. Previous studies have established the two determinant status in rabbits and guinea pigs yet, the supporting evidence was still lacking in the murine system. Therefore, further experiments were carried out to: confirm the two determinant status in mice, determine the ratio of N-versus C-specific antibody in various murine strains, and to eventually dissect the role of each determinant at the genetic level.

I. Choice of molecular probes

At first, an array of synthetic peptides representing either of the two determinants was used to determine the specificity of anti-Fd antibody. It was found however, that extremely high concentrations (up to 1000-fold higher molar ratio of peptide to Fd) were needed in hapten inhibition of the ELISA to obtain a determinant-specific value (direct binding assays could not be performed). This observation corroborates with others made in some systems where the use of small peptides
Figure 9. The amino acid sequence of Fd. The two major antigenic determinants are indicated. The sites of the selective enzymatic cleavages used for production of unideterminant molecules are shown.
TRYPSIN

\[ \text{ALA-TYR-LYS-ILE-ALA-ASP-SER-CYS-VAL-SER-CYS-GLY-ALA-CYS-ALA-ASP-VAL-ASN-ALA-Ile} \]

\[ \text{SER} \quad \text{GLN} \quad \text{GLY} \quad \text{ASP} \quad \text{SER} \quad \text{ILE} \quad \text{PHE} \quad \text{VAL} \quad \text{ILE} \]

THE N-DETERMINANT

\[ \text{GLU-GLN-VAL-PRO-ALA-GLY-VAL-PRO-CYS-VAL-ASN-ALA-CYS-ASN-GLY-CYS-ASP-Ile-CYS-THR-ASP-ALA-ASP} \]

CARBOXYPEPTIDASE A

THE C-DETERMINANT
demonstrated up to 4000-fold decrease in antibody binding as compared to the native sequence (120,215,238). Therefore, it became clear, that if reproducible values were to be obtained for the ratio of reactivity to the two determinants of Fd, a more reliable route had to be found.

Ideally, probes were needed which are similar in size to Fd yet only possess one functional antigenic determinant. It was felt that enzymatically cleaved native Fd, devoid of either of the two determinants would be preferable in determining the specificity of anti-Fd antibody. The benefits of this approach would be two-fold: (1) the ELISA could be performed by directly binding the enzyme fragments to the substrate plate thus, permitting not only the generation of "hapten" inhibition data, but also solid phase reactivity, (2) the putatively unideterminant molecules could be used in vivo, possibly without the use of carrier molecules, which may obscure immunoregulatory studies. For these purposes, it would be necessary to have enzymatic fragments with the following properties: (1) the resultant molecules should be large enough to bind adequately to ELISA plates, and, (2) the fragments carrying only one intact determinant should be readily purified from the digest.

II. Choice of enzymes

The enzymes that were chosen to satisfy the criteria outlined above were trypsin and carboxypeptidase A. Trypsin has one available cleavage site in Fd as demonstrated in Figure 9. The cleavage of the N-terminal tripeptide results upon dialysis in a molecule 52 amino acid residues in length with a functional C-determinant; this molecule is to be subsequently called "C".
Carboxypeptidase A, an exopeptidase, sequentially digests the carboxy-terminus of Fd, cleaving off the terminal glutamic acid followed by the glutamine. The enzyme is inhibited by the presence of the proline in the penultimate position, yielding a 53 amino acid molecule with a functional N-determinant; this molecule is subsequently referred to as "N". A complicating factor in the carboxypeptidase A (CPA) digest of Fd is the charged terminal glutamic acid, which is a very poor substrate for CPA due to its charge at pH 7.5, the pH optimum of CPA. It has been suggested (250,251), that lowering the pH to 5 increases the rate of glutamic acid cleavage, however, this tends to inactivate the enzyme. This unfortunate situation necessitates the use of a rather high amount (up to 50% w/w) of total CPA to obtain a complete digest. These problems were encountered by Tanaka et al. (248), as digestion with CPA originally yielded an improper C-terminal sequence of val-ser-glu-COOH.

Carboxypeptidase Y (CPY) had also been tried as it has a broader amino acid specificity including proline and is inhibited at glycine (one is found at residue 50, an ideal stop for cleavage). However, CPY is a much slower-acting enzyme than CPA and is also impeded by pro and glu (that is, the rate of removal of a terminal glu or pro is 50-times slower than that of val). In our hands, no measurable digestion was observed with CPY. Carboxypeptidase B (CPB) was totally unacceptable in this task because of its preference for hydrolysis of basic residues such as lysine and arginine.
Assessment of the completeness of the enzymatic digests was mandatory if these molecules were to be used successfully as antigenic probes. Not only would most biochemical techniques necessitate purification of the digests at every step, but also, the sensitivity of most of these techniques is too low to measure contamination of undigested Fd at levels where antibodies would detect them. Thus, immunochemical techniques were developed for the characterization of these fragments. Monoclonal antibodies by definition react with one determinant on an antigen, hence their use would constitute a highly sensitive and potentially quantitative technique in determining the extent and completeness of the digest: for a particular monoclonal, digestion with one enzyme should be reflected in a decrease of reactivity while no change ought to be observed with the other digest.

The enzymatic digests were characterized with the use of anti-Fd monoclonal antibodies produced in the laboratory by Michael Weaver (6-90 and 17-3) and Doug Demetrick (DFd-2E). Figures 10, 11a and 11b demonstrate the reactivity of these monoclonals, both by hapten inhibition and in a standard ELISA. Figure 10 demonstrates 6-90 in the "hapten" inhibition assay using Fd, "N" and "C", which have been dialyzed as 6-90 is extremely sensitive to 2-mercaptoethanol ($10^{-6}$ M) present in the digests. Dialysis was also necessary because the tripeptide produced by trypsin digestion was found capable, despite its small size, of inhibiting 6-90 binding to Fd on the plate. This assay demonstrates the absolute specificity of 6-90 for the N-determinant. Figure 11 demonstrates the
Figure 10. Hapten inhibition assay with 6-90. Affinity purified 6-90 was incubated overnight with dilutions of native Fd, (A) "N"-fragment (©) or "C"-fragment (-visible) and tested on ELISA plates coated with native Fd at 4.0 µg/ml. The test was performed in quadruplicate.
Figure 11. Solid phase ELISA with A) DFd 2E and B) 17-3. Monoclonal antibodies DFd 2E and 17-3 were tested on ELISA at a concentration of 0.5 μg/ml of affinity-purified antibody. The plate was coated with 5.0 μg/ml of Fd, "N" or "C". The test was performed in quadruplicate and demonstrate the inability of DFd 2E and 17-3 to bind "N", even at 5.0 μg/ml which constitutes a level of 5- to 10-fold antigen excess.
reactivity of a) DFd-2E and b) 17-3 with "C" and Fd but not "N" when these molecules are bound to the substrate plates. It must be pointed out here that monoclonal antibodies have very restrictive antigen binding requirements. For example, 6-90 does not react with either fragment when present as the solid phase thus, necessitating analysis in the liquid phase in the hapten inhibition, or blocking assay. Conversely, both DFd-2E and 17-3 show very poor inhibition of binding, even with the native molecule in hapten inhibition reactions, therefore the solid phase assay is the only one of value for these monoclonals. Therefore, with these two assays and these three monoclonals, the purity of the digests can be assessed and the fragments quantified.

In the interest of maintaining an accurate measurement of the concentration of fragments throughout all the experiments, the fragments were not purified from the contaminating enzymes on a routine basis. Where such purification was deemed necessary, techniques were selected for this purpose; these are described in Appendix III. Since the sera that were to be tested for "N" and "C" reactivity originate from mice immunized with the native molecule and not the fragments themselves, there was no expected interference in the use of unpurified preparations.

IV. Specificity of anti-Fd sera

After the purity of the fragments was assessed, a high responder (B10.BR) anti-Fd serum was tested with the fragments both in the blocking (inhibition) assay, Figure 12, and solid phase ELISA, Figure 13. It was observed that 10 to 30 percent of the antibody was "N" directed, the rest being "C" directed, depending on the assay. What these two figures also
Figure 12. Hapten inhibition assay with B10.BR anti–Fd antiserum. B10.BR anti–Fd serum was incubated at a final dilution of 1:200 with dilutions of Fd, "N" and "C" and assayed on an ELISA plate coated with native Fd at 4.0 µg/ml. The test was carried out in quadruplicate.
Figure 13. Solid phase ELISA with B10.BR anti-Fd sera. Five individual sera from Fd-immunized B10.BR mice were tested on the standard ELISA on Fd, "N", and "C" bound to the plate at 5 μg/ml. Colour development on the "N" and "C" fragment ELISA were added for each serum to give the "N + C" value. Each test was performed in quadruplicate. Average values ± S.E. are shown.
demonstrate is that "N" reactivity added onto "C" reactivity adds up to the reactivity to the native molecule, indicating, albeit still indirectly, that these two determinants represent the only two antigenic moieties of Fd. This ratio of "N" to "C" reactivity was observed to be a stable characteristic: in Figure 12, the ratio is constant at each concentration of blocking fragment. In addition, when the anti-Fd serum was titrated in the solid phase assay as seen in Figure 14, two lines of evidence are added in support of the stability of this N:C ratio: (a) the ratio is the same at any dilution tested, and (b) when the titre of the serum was calculated for each fragment, there was a 4-fold difference in reactivity supporting a 25:75 "N" to "C" ratio. When comparing the solid phase and blocking assays one finds that generally lower N-reactivity values are obtained with the solid phase assay, however, this discrepancy is uniform for all sera tested and in all probability reflects altered binding properties of the fragments. Although the blocking assay is probably the more accurate one in describing the reactivity ratio (N:C), it is a more cumbersome assay with a requirement for larger quantities of both sera and enzyme fragments, and therefore not used at all times, particularly when extensive surveys of individual sera were being run.

As the stability of the N:C ratio was assessed for a single serum, the question was raised as to the conservation of this ratio within sera from mice of the same or different strains i.e. is the ratio random from mouse to mouse or is it a constant feature? Individual sera from B10.BR (both 2° and 3° bleeds were tested), B10.S and C57BL/10 mice were
Figure 14. Titration of B10.BR anti-Fd serum on "N" and "C" fragments. Pooled B10.BR anti-Fd serum was titrated from 1:100 to 1:12800 on 4 µg/ml of "N" or "C" fragment (The higher curve describes binding to "C", while the lower one binding to "N"). Arrows indicate end point of titration on both antigens. The 4-fold difference represents roughly the characteristic ratio of N : C antibody.
<table>
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<tr>
<th>Serum Dilution</th>
<th>N:C Ratio</th>
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<tbody>
<tr>
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<tr>
<td>1/400</td>
<td>19.5</td>
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<td>1/800</td>
<td>19.7</td>
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<tr>
<td>1/1600</td>
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<td>1/3200</td>
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<td>1/6400</td>
<td></td>
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<tr>
<td>1/12800</td>
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Figure 15. % "N" reactivity of individual sera. Individual sera from B10.BR (2° and 3° responses), C57BL/10 (2°) and B10.S (2°) mice immunized with Fd. Each dot represents results obtained from the solid phase ELISA using Fd, "N" and "C" at 1 μg/ml. In each case, the bar represents the mean of the response within a strain.
tested for fragment reactivity in the solid phase assay and the results of these assays are shown in Figure 15. It can be readily seen that the ratio is consistent within a strain and is maintained throughout the course of the response as demonstrated with B10.BR secondary (2°) and tertiary (3°) sera. It was evident that although most mice within a strain mount a characteristic specificity of the anti-Fd response, variations exist from strain to strain. The next step was to look for a genetic basis for the determinant specificity.

V. Genetics of determinant specificity

At first, N:C ratios were tested in all available H-2^k strains to determine whether the ratio is genetically encoded by MHC or non-MHC genes. Figure 16 demonstrates the results obtained with eight H-2^k strains as compared to two H-2^s strains; clearly all H-2^k mice produce 5-20% N-reactive antibody whereas H-2^s mice make 40-50% N-specific antibody. It was then of interest to see whether this determinant specificity was controlled by K/I-A, as was the magnitude of the response. Figure 17 indicates that indeed this is the case as all high responder mice demonstrate the characteristic high anti-"C" response of the B10.BR mice, whereas all intermediate responding strains make considerably less C-specific antibody relative to the high responders. Depending on the assay used, it can be calculated that Fd-immune high responders make between 1.2 - 3.6 μg/ml N-specific antibody and intermediate responders make between 1.6 - 2.0 μg/ml N-specific antibody. However, when C-specific antibody is quantified, high responders make 8.4 - 10.8 μg/ml as compared to 2.0 - 2.4 μg/ml in
Figure 16. N : C ratio of H-2\textsuperscript{k} strains of varying allotypes.

Fd-immune sera from eight H-2\textsuperscript{k} strains of mice varying in allotype at IgCH were compared for their % "N" -reactivity using the solid-phase ELISA with Fd, "N" and "C" at 4 μg/ml. The results were compared to two H-2\textsuperscript{S} strains of mice. Values in the bar graphs are the averages of seven sera per strain ± S.E. For each strain, the responder status is shown. The symbols HR refers to high-responder strains and IR refers to intermediate responders to Fd.
<table>
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<tr>
<th>STRAIN</th>
<th>RESPONDER STATUS</th>
<th>HAPLOTYP</th>
<th>IgCH ALLOTYP</th>
<th>% N-REACTIVITY</th>
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<tbody>
<tr>
<td>ST</td>
<td>HR</td>
<td>k</td>
<td>a</td>
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<tr>
<td>C58</td>
<td>HR</td>
<td>k</td>
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<tr>
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<td>HR</td>
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<td>IR</td>
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<tr>
<td>SJL</td>
<td>IR</td>
<td>s</td>
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Figure 17. N : C ratio of recombinant strains of mice on the B10 background. Fd-immune sera from recombinant mice on the B10 background were titrated and, the dilution of serum giving a reading of 0.5 at A405nm after 60 min on ELISAs done with 4 μg/ml native Fd bound to the plate was chosen for the "hapten- inhibition" assay. Each serum was tested in the inhibition assay with 0.5, 1.0 and 2.0 μg/ml of the "N" and "C" fragments. For each serum the % "N"-reactivity obtained at each dilution of fragment was calculated and the values for the three levels were averaged. Values in the bar graphs are the averages of six sera per strain ± S.E. For each strain, the responder status is shown [HR = high responder; IR = intermediate responder; LR = low responder].
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<td><strong>B10A(3R)</strong></td>
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<td><strong>B10A(5R)</strong></td>
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<td><strong>B10S</strong></td>
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<td><strong>B10S(9R)</strong></td>
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<td><strong>B10,HTT</strong></td>
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**Diagram:**

- **K A B J E C S G D**
- **B10.8R**
- **B10A**
- **B10A(2R)**
- **B10A(4R)**
- **C57BL/10**
- **B10A(3R)**
- **B10A(5R)**
- **B10S**
- **B10S(9R)**
- **B10,HTT**

**Legend:**
- **HR:** High Reactivity
- **IR:** Intermediate Reactivity
- **LR:** Low Reactivity
intermediate responders, indicating about a four-fold increase in C-specific antibody in high responders as compared to the intermediate responders.

Figure 17 provides an additional interesting piece of information concerning the B10.HTT strain which was found previously to show results anomalous with the mapping studies. When some of these low responding sera were tested (incubation was extended to 18-24 hours in order to allow sufficient colour development), it was found that they demonstrated the same determinant specificity as other I-A$^s$ mice [B10.S and B10.S(9R)], indicating that although a defect exists at the level of the magnitude of the response, the gene conferring the specificity of the response is intact in this strain.

VI. The F$_1$ response

Additional information on the genetics of the response to Fd was obtained from limited studies with F$_1$ strains of mice. Figure 18 demonstrates the results obtained with the F$_1$ strains as compared to the parentals. In the analysis of the magnitude, it can be readily seen that there is a gene dose effect. The (B10.BR x B10.HTT)F$_1$ strain conforms to the standard gene dose effect, suggesting that if the B10.HTT strain induces a suppressor phenomenon through a possible I-A$^s$ - I-C$^k$ interaction, it is one that is not active in trans. The specificity of the antibody response was totally that of the higher responding parent, at first suggesting that although the magnitude of the response was controlled by a gene dose phenomenon, the specificity of the response was controlled by an allelically excluded dominant gene phenomenon. The only
Figure 18. Determinant specificity and magnitude of the responses of F1 mice as compared to parental strains. Solid phase ELISAs on A) Fd at 4 μg/ml and B) "N" and "C" at 4 μg/ml describe A) the magnitude and B) the specificity (N : C ratio) of the response of F1 mice as compared to the parental strain. A minimum of 6 mice were used per assay ± S.E.
STRAIN

BIO.BR
BIO.D2
(BIO.BR x BIO.D2)F1
CBA
DBA/2
(CBA x DBA/2)F1
C57BL/10
DBA/2
B4D2F1
BIO.BR
BIO.S
(BIO.BR x BIO.S)F1
BIO.BR
BIO.HTT
(BIO.BR x BIO.HTT)F1

(A) MAGNITUDE: Absorbance at 405 nm

(B) SPECIFICITY: %anti-N

0 0.5 1.0 1.5

0 10 20 30 40 50 60
that could be used to support this hypothesis was the 
(B10.BR x B10.S)F₁ strain as the high responder makes 9.3% N and the 
intermediate responder, B10.S makes 45.2% N. Therefore, the prediction 
could be made that if there is a dominance phenomenon (i.e. the 
specificity of the response is that of the higher responding percent) for 
specificity one would observed 9.3% N, but if a gene dose phenomenon (i.e. 
the specificity of the antibody is dictated by the contributing percentage 
of each chromosome) regulates the magnitude as well as the specificity, it 
was calculated that the % N reactivity could be as low as 18.5%. The 
actual value that was obtained was 13.5%, exactly in between the two 
calculated values. As the limits of this assay cannot distinguish between 
these two possibilities, it is safest to assume that the gene dose 
phenomenon accounts for the specificity as well.

VII. The "M" molecule

Up to this point, the two determinant status of the response in mice 
to Fd had been arrived at indirectly by adding the N-reactivity to the 
C-reactivity and obtaining roughly the sum of the anti-Fd value (refer to 
Figures 12 and 13). However, a third determinant had not been formally 
excluded. For this purpose, the "M"-fragment ("M" for middle) was 
produced by a double digestion of native Fd; i.e. trypsin digestion 
followed the completed CPA digest. Results shown in Figure 19 using the 
solid phase assay and Figure 20 using the blocking assay demonstrate not 
only that a third antibody-reactive determinant is not present, but that 
the "stubs" of the determinants left over after the digests do not react 
with any anti-Fd antibody. Therefore, the conclusion can be drawn that
Figure 19. Reactivity of anti-Fd sera with "M. B10.BR (1:1000), B10.S (1:200) and C57BL/10 (1:200) pooled Fd-immune sera were tested on Fd and the doubly digested fragment "M" at 2 μg/ml. The test was performed in quadruplicate.
Antigen: STRAIN: SERUM DILUTION:

Fd M BIO-BR 1:1000

Fd M BIO-S 1:200

Fd M C57 BL/10 1:200

Absorbance at 405 nm
Figure 20. Hapten inhibition of anti-Fd serum with "M. Pooled B10.BR anti-Fd serum (1:1000) was incubated overnight with 0.5, 1.0, 2.0 and 4.0 µg/ml Fd or "M" and assayed on ELISA plates coated with Fd at 2 µg/ml.
not only is the magnitude of the anti-Fd response controlled by I-A

gene(s), but also the control of determinant specificity to the only two
determinants of Fd is under the influence of this subregion.

VIII. Conclusion

In summary, it was found that the most amenable probes for analysis
of anti-Fd antibody specificity are not synthetic peptides, but fragments
of the native molecule. This can be explained by the fact that by the
simple removal of two or three amino acids, essentially monodeterminant
molecules are generated which have physiochemical properties very close
but not identical to the native molecule. Therefore, these molecules may
be bound to ELISA plates for direct binding analysis of anti-Fd sera, as
well as used in blocking assays in concentrations totally comparable to
the ones used with native Fd. It was found that all anti-Fd reactivity
could be accounted for by adding "N" and "C" reactivities. Lack of
reactivity with "M" supported very strongly the two determinant model at
the antibody level. Not only are the relative reactivities to the two
determinants conserved within all mice within a strain, but strain
differences are seen to correlate to the genetics of the response mapping
to K/I-A. All high responders make predominantly (70-90%) C-reactive
antibody while all intermediate responders generate roughly equivalent
amounts of anti-"N" and anti-"C" antibody. Since one can calculate that
the amount of anti-"N" antibody synthesized is roughly equivalent by both
phenotypes of the response, it may be stated that the net effect of I-A
genes is to promote an increase in C-specific antibody.
Results with the aberrant B10.HTT strain demonstrate that the information necessary for selecting a 50:50 N:C response to the two determinants is present, indicating that the lesion is at the level of promotion of the antibody production and not at the level of I-A regulated determinant selection.
Chapter 5. Results: Fragments of Fd: their immunogenicity and role in cellular interactions

I. T-cell specificity

As the specificity of anti-Fd antibody had been assessed, the next step was to determine whether T-cells immune to Fd had a similar or different specificity. Fd-immune B10.BR lymph node cells were set up in the proliferation assay with Fd, "N", "C" and "M"; KLH immune mice served as the specificity control. Figure 21 describes these data while Figure 22 demonstrates antigen dose response curves. It can be readily seen that "C" seems to stimulate Fd-immune T-cells to the same extent as the native antigen while both "N" and "M" induce roughly a two-fold lower proliferative response. These data suggest two things: a) that the T-cell specificity of Fd-immune cells is similar to the antibody specificity (C-reactivity greater than N-reactivity) and b) that sequences within "M" are still immunostimulatory at the T-cell level where there is no observable reactivity at the antibody level. This dichotomy in results warranted further work with these "monodeterminant" molecules, and their activity in vivo was assessed.

II. Preliminary in vivo studies: the antibody response to fragments

In a pilot study, B10.BR mice were immunized with "N" and "C" fragments which had been generated through standard digestion procedures followed by G-50 purification. Despite the fact that there was
Figure 21. Proliferation of Fd-immune lymph node cells to Fd, "N", "C" and "M". Fd-immune B10.BR (primed with 50 μg Fd, rested 30 days, boosted with 50 μg Fd, sacrificed 7 days later) pooled lymph nodes were assayed for proliferation as described in figure 2. Fd, "N", "C" and "M" antigens (open bars) in culture were all at 50 μg/ml final concentration. KLH-immune mice were used as control, as was KLH in the cultures (shaded bars). Values are cpm x 10^4 ± S.E.
Figure 22. Antigen titration of the proliferative response of Fd-immune lymph node cells to Fd, "N", "C" and "M". Culture conditions were as described in figure 21. Fd "N", "C", "M" (open bars) and "KLH" (hatched bars) were used at 12.5, 25.0, 50.0 and 100.0 μg/ml final concentration.
considerable difficulty in characterizing and quantitating the fragments, rather interesting results were obtained. Figure 23 demonstrates both primary and secondary responses to immunization with 50 µg Fd, "N" or "C" as well as mixtures of these three antigens (50 µg each). It can be seen that although the "N" and "C" fragments in this case induce little, if any, antibody they have an effect on the Fd response. Adding 50 µg of the "N" molecule to 50 µg of native Fd for immunization decreases the primary anti-Fd response by more than half. In the secondary response, the "N" + Fd response is still significantly lower than the Fd response. In the primary response, addition of "C" to Fd had no effect, while in the secondary response, this Fd + "C" response is higher than the Fd response. When the N:C ratios of these anti-Fd responses were calculated, some rather interesting results were obtained. The results are shown in Figure 24. Substantial differences, especially evident in the 7 day bleed, are seen and lead to the following postulations: Since the Fd and "N" immunized animals have very similar results, the response may be due to a contaminant, however, co-immunization with Fd + "N" leads to a very low reactivity to "N" which would suggest that the inhibition generated by co-immunization shuts down the anti-N response preferentially. On the other hand, the decreased N:C ratio in Fd + "C" immunized animals as compared to Fd-immunized mice may reflect a net anti-C help manifested in the increased overall response to Fd + "C". The 50:50 response of "N" + "C" immune mice may reflect the truncation of signals that regulate the specificity of the response.
Figure 23. Immunogenicity of fragments and effects of co-immunization.

B10.BR mice were immunized with 50 µg Fd of G-50 purified "N" and "C" fragments or a combination of two of the above antigen (50 µg of each). The primary response was tested on ELISA coated plates at 1 µg/ml and serum at 1:100 dilution 14 days after the immunization. Secondary responses were tested in a similar fashion 7 and 18 days after the boost. Results represent the average of six animals per group ± S.E.
PRIMARY RESPONSE: 14 day
SECONDARY RESPONSE: 7 day
SECONDARY RESPONSE: 18 day
Figure 24. Determinant-specificity of fragment-immune B10.BR mice. The secondary response sera (7 and 18 day) of fragment-immune B10.BR mice described in figure 23 were tested for determinant specificity on ELISA plates coated with "N" and "C" at 1 µg/ml. Sera were tested at 1:100. Values represent sera with an absorbance of 0.1 at $A_{405nm}$ as tested in figure 23 (± S.E.). C responses are not applicable (N/A) as these mice did not make any antibody.
7 day bleed

Fd
N
C
Fd + N
Fd + C
N + C

18 day bleed

Fd
N
C
Fd + N
Fd + C
N + C

% "N" Reactivity
Although these observations are interesting, the explanations are speculative, as this experiment was not repeated. The following experiments all differed from these as the fragments were not purified on G-50; therefore, the concentrations were known at all times. As will be seen, the newer fragments induced a different pattern of responsiveness.

III. Responses of B10 congenic mice to fragments

B10.BR, C57BL/10 and B10.D2 mice were immunized with Fd, "N", "C" and "M" in order to assess the immunogenicity of these fragments both at the antibody and T-cell proliferative levels with respect to H-2-linked control. Although a large amount of data was generated from these experiments (all mice were tested at 14, 19, 28, 35 days after the primary immunization) in the interest of making succinct comparisons, three figures will be presented: Figure 25 summarizes 18-22 day primary antibody responses to Fd and the fragments, Figure 26, the 7 day secondary antibody responses and Figure 27, the results of T-cell proliferative assays. The results obtained with each immunizing antigen will be addressed separately.

A. Response to Fd.

In order to confirm previous results and to provide a baseline for comparisons, all three strains of mice were immunized with Fd or saline as control. The low primary response is increased by the secondary stimulus, yet the pattern is constant: B10.BR mice make the largest anti-Fd response, followed by the intermediate status of the C57BL/10 mice, and B10.D2 mice make no anti-Fd antibody. With respect to the proliferation assay, only B10.BR mice demonstrate a
Figure 25. Immunogenicity of "N", "C" and "M" in H-2\textsuperscript{k}, H-2\textsuperscript{b} and H-2\textsuperscript{d} mice: primary response. a) B10.BR, b) C57BL/10 and c) B10.D2 mice were immunized with Fd or unpurified "N", "C" and "M" fragments. 18-22 day bleeds were tested on ELISA plates coated with Fd at 4 µg/ml. 10 mice were used per group. Average is shown ± S.E. Sera were tested at 1:100 and the assay developed for 1.0 hr. N.I. = animals not immunized with fragments.
Figure 26. Immunogenicity of "N", "C" and "M" in H-2^k, H-2^b and H-2^d mice: secondary response. a) B10.BR, b) C57BL/10 and c) B10.D2 mice were immunized as described in figure 25, rested a minimum of 30 days, boosted in the identical manner and bled 7 days later. The ELISAs were performed with Fd coating the plates at 4 µg/ml, the sera at a 1:250 dilution and the reaction recorded after 30 min. 10 mice were used per group, averaged results ± S.E. are shown. N.I. = animals not immunized with fragments.
Figure 27. Proliferative response of Fd-, "N"-, "C"-, and "M"- immune B10.BR, C57BL/10 and B10.D2 mice to Fd. Proliferative assays were carried out as described in figure 2 with mice used in figure 26. Immune lymph node cells from Fd-, "N"- "C"- and "M"- immune a) B10.BR, b) C57BL/10 and c) B10.D2 mice were cultured with native Fd at a final concentration of 50 µg/ml. Average net cpm are shown ± S.E. N.I. = animals not immunized with fragments.
significant lymphoproliferative response to stimulus with Fd \textit{in vitro}. An extremely low proliferative response with C57BL/10 and no proliferation with B10.D2 lymph node cells totally supports previous observations.

B. \textbf{Response to "N".}

When "N" is used as immunizing antigen, an interesting pattern of immunogenicity is observed in the high responder mice. Although only a very low antibody response is seen with this antigen, "N" stimulates a proliferative response equalling the one seen by Fd-immune lymph node cells. What this result suggests is that the determinant responsible for priming of proliferation is present in "N" while the one inducing antibody is possibly absent. This would agree with the ratio of reactivity of B10.BR sera: a predominantly C-specific response is seen indicating that the carboxy terminal two amino acids are intrinsic in the activity of the C-determinant.

"N" was found to be non-immunogenic in C57BL/10 and B10.D2 mice at the antibody level. Since both of these strains are low and non-proliferators to Fd, it was not surprising to find that "N" did not induce an appreciable \textit{in vitro} response. It may therefore be concluded that the "N" molecule provides a T-cell stimulatory signal yet on its own does not induce significant antibody production.

C. \textbf{Response to "C".}

Immunization with "C" provided the most interesting results. In both primary and secondary responses to "C", all three strains of mice gave significantly higher responses to Fd than they did after immunization with the native antigen. In fact, immunization with "C" turns the B10.D2
non-responder into a responder. This data is the most supportive of the proposal that a suppressor determinant resides in the amino-terminal portion of Fd. Cleavage of the terminal amino tripeptide relieves the "suppressive" effects most strongly exerted in B10.D2 mice. This possible suppressive effect is also seen in C57BL/10 mice as well as B10.BR high responder mice.

When "C" immune lymph node cells were used in the proliferation assay with native Fd as stimulus, it was interesting to observe that although "C" primes for a very high antibody response, it is a poor inducer of proliferating T-cells in all strains tested.

It could not induce proliferation in either of the non-proliferating strains (C57BL/10 and B10.D2). These results therefore indicate not only that "C" lacks the powerful T-cell stimulator sequence of "N" and Fd, but also that, as previously proposed, there is a lack of correlation between antibody production and T-cell proliferation.

D. Response to "M".

As was seen above, anti-Fd antibody has no reactivity with "M", indicating that antibody binding involves the terminal residues cleaved-off during production of this fragment. Results of proliferative data however, indicated that like "N", "M", is capable of inducing in vitro, Fd-immune B10.BR T-cells to proliferate. When "M" was used as an immunogen, the dichotomy between antibody and proliferative results was repeated. None of the sera tested, primary or secondary, B10.BR, C57BL/10 or B10.D2, reacted with Fd in the ELISA. However, "M" does prime for a proliferating cell population (as does "C") in the high responder strain,
indicating that T-specific sequences exist in "M", which are recognized both in vivo and in vitro.

The following conclusions may be made concerning the immunogenicity of "N", "M" and "C": 1) The "N" molecule contains two major properties (a) it has the in vivo ability of priming for a proliferating cell and (b) it is a poor stimulator of anti-Fd antibody. 2) The "C" molecule may lack a suppressor determinant as it turns an Fd non-responder animal into an antibody producer but not a proliferator. "C" is a poor inducer of the proliferative response in high responders indicating a lack of correlation between antibody production and proliferation. 3) The "M" molecule is inert at the antibody level but has sequences responsible for T-cell proliferation. 4) With respect to the proliferative assay, "N" primes for a good Fd-specific proliferative response while Fd primed cells do not proliferate well in the N-specific response. The converse situation exists with the "C" molecule indicating that events involved in the in vivo priming with Fd as compared to the "N" and "C" fragments are distinct from the events controlling the in vitro proliferative response. This observation suggests that regulatory sequences are found at both termini of Fd, and that truncation of the C-terminus affects the priming of a proliferative population while truncation of the N-terminus affects the actual in vitro stimulation of the primed population.

IV. Response of A.SW mice

As there was a consistent lack of correlation between proliferative and antibody responses of C57BL/10 mice, a proliferating intermediate
responder was tested in order to determine the effect of "N", "C" and "M" priming of the proliferative response. The results obtained with A.SW mice (H-2^s) are described in Figures 28A and B, and 29, and provide the following information: A.SW mice, as predicted, do proliferate to stimulation with Fd, and in the expected range (compare to SJL and B10.S results, Figure 2). The "N" fragment may or may not induce a proliferating cell; this depends on how the assay is analyzed. Personal bias would favour the net counts results that indicate that priming of the proliferative response is present. In both Figures 28A and B, the proliferative response by "M"- and "C"-primed mice is very poor and not much above non-immune control.

The antibody data of Figure 29 provide anomalous results: H-2^s mice were characterized as intermediate antibody producers, yet A.SW demonstrates no antibody production after immunization with the native molecule (although an appropriate proliferative response is observed in these same mice). No response was observed with "N" and "M", while all "C" immunized mice produced an antibody level comparable to the one observed in the non-responder, B10.D2 mice.

Unfortunately, this study with A.SW mice has not added much positive information clarifying the immunoregulatory properties of the fragments of Fd. Yet, it may be clearly stated that this is yet another piece of supporting evidence where the two assays of immunity do not correlate.
Here, there exists a situation where a strain proliferates to native Fd, and possibly "N", with a total absence of antibody, and lacks a
Figure 28. The proliferative response of A.SW mice. A.SW mice were immunized according to the protocol described in previous figures and the assay carried out as in figure 2. Fd-, "N"-, "C"- and "M"- immune lymph node cells were cultured with Fd at 50 μg/ml. Ten mice were tested in each group and values representing A) net counts and B) the stimulation index include the average ± S.E.
Figure 29. The antibody response of fragment-immune A.SW mice. A.SW mice primed with Fd, "N", "C", and "M" were boosted with the same antigens after a 30 day rest, and tested for antibody production 7 days after the secondary immunization. The ELISA (plates coated with Fd at 4 μg/ml) results are expressed as units of absorbance at 405nm ± S.E. 10 mice were used in each group.
ABSORBANCE at 405 nm
proliferative capacity in animals immune for antibody production ("C" fragment mice).

Explanations for these observations of discrepancy with the A.SW anti-Fd response are lacking at this time as H-2^s intermediate responders carry the A.SW H-2. The suggestion can be made, that future work in this area should involve the analysis of A.SW mice in conjunction with SJL, or more appropriately Bl0.S mice.

V. Conclusion

In summary, in the study of the immunogenicity of the "N", "C" and "M" molecules a few general proposals may be put forward regarding the immunodominant and immunoregulatory properties of the determinants of Fd. It is clear that the C-determinant is immunodominant with respect to B-cell activity (antigen recognition and antibody production) as well as at the T-cell level (as measured by proliferation). This is evident from the observation that although "C" cannot prime for an optimal Fd-specific proliferative response, it equals the Fd-specific response, of Fd-immune T-cells in culture.

The N-determinant, on the other hand, demonstrates a very poor T-cell reactivity with Fd-primed cells in vitro, and it also has a low capacity for inducing an antibody response. However, the N-determinant provides the stimulus for immunoregulation of both the antibody and proliferative responses. Two lines of evidence to date indicate that the amino-terminal tripeptide may induce specific suppression: a) immunization with "C" (devoid of this "suppressive" tripeptide) induces a very high anti-Fd response at the antibody level, even in Fd
non-responders (B10.D2 and A.SW) and b) an early unconfirmed study involving "N" co-immunization with Fd decreases the antibody response to Fd substantially.

Another major observation is made in this chapter: there is no correlation between the proliferative assay and antibody production in the Fd system. In fact, an inverse relationship seems to exist. In B10.BR mice, high proliferation to Fd induced by "N" is paralleled by low antibody production, poor induction of proliferation by "C" is mirrored by good antibody production. C57BL/10 non-proliferators are intermediate antibody producers, while A.SW proliferators make no anti-Fd antibody, unless they are stimulated with "C", in which case they do not proliferate. Non-proliferators and non-antibody producers, B10.D2 mice, make a good anti-C antibody response with no concomitant proliferation.

Ferredoxin, therefore, despite a few anomalies in the results, is a very elegant probe of immunoregulation: both the antibody and proliferative responses can be dissected at the single determinant level with a number of resulting observations that put in question some of the previous interpretations of the measures of immunity.

As both the genetics and immunoregulatory properties of the Fd antigenic determinants are at least partially understood, ongoing and future research is needed to define the cells involved in the regulatory phenomena described above. A number of experiments have been carried out that attempted to further define the basis of non-responsiveness to Fd. This work is covered in Chapter 6 and in Appendices VI and VII.
Chapter 6. Results: Insights; Nature of Control

This chapter deals with experiments that were set up to confirm the presence of a "suppressor" determinant; in the previous chapter, evidence was obtained implicating the amino-terminal tripeptide of the N-determinant as part of an immunoregulatory signal that down-regulates the antibody response to Fd. The identity of the cell that recognizes this determinant and induces a decrease in antibody production is not known. In order to elucidate the nature (i.e. helper versus suppressor) of the cells primed with the fragments, adoptive transfer experiments were carried out.

Figures 30A and B, 31 and 32A and B represent the results of four adoptive transfer experiments. Each of these will be described separately as they were carried out under variable protocols. All experiments were assayed at least 14 days post challenge, these results are shown.

I. Adoptive transfer I: B10.BR T- and B-cell transfers with the use of the panning technique.

Figure 30A demonstrates the results from the first adoptive transfer. B10.BR mice that had been primed with Fd or the fragments, were rested for 75 days, boosted with the same antigens and sacrificed 7 days later. Three animals (donors) were used per group. T- and B-cell populations were enriched by panning (259). Briefly, petri plates (Falcon 3001), preincubated for 2 days with PBS at 4°C, were coated for 24 hr at 4°C
Figure 30. Adoptive transfers I and II. A) B10.BR mice primed and boosted with Fd, "N", "C", and "M" were rested 7 days and sacrificed. Spleens from 3 donors per group were panned on coated plates (10 μg/ml RaMig) and T- and B- cell enriched populations were isolated: 2 hour non-adherent cells were collected for the T-enriched population while adherent cells were scraped off with teflon policemen for the B-enriched population. 5 x 10^6 immune T- and B- cells were transfered i.v. to irradiated (450R) recipients in the indicated combinations. Two to six mice were used per group; they were challenged with 50 μg Fd/CFA 7 days later. Mice were bled and tested on Fd coated ELISA plates 14 days after the challenge. Average absorbance at 405nm ± S.E. is shown. B) Adoptive transfer II was carried out as adoptive transfer I with the exception that T- and B- cells were enriched by nylon-wool and αThy 1 + C treatment. As more cells were recovered, all recipient mice (5 per group) received 1 x 10^7 immune T- and B- cells.
T cells immune to: Fd Fd M M N N C C
B cells immune to: Fd M Fd M N C N C
number of mice: 2 6 2 6 3 5 3 5

(A) Absorbance at 405 nm

(B) Absorbance at 405 nm
Figure 31. Adoptive transfer III and the effect of irradiation of recipients. The adoptive transfer was performed as described in figure 30 with a few exceptions: for irradiated recipients, immune T-cells and non-immune B-cells were employed, while non-irradiated recipients received only immune T-cells. The ELISA results of donor mice are shown for comparison.
Figure 32. **Adoptive transfer IV** - C57BL/10 mice primed and boosted with Fd or the fragments were rested 45 days before use as donors. Immune T-cells were enriched by nylon wool and transferred to irradiated recipients. $1 \times 10^7$ and $2 \times 10^7$ immune T-cells were transferred as well as mixtures of immune T-cells. At all times, equal numbers of B-cells (non-immune) were used. 14 day sera from (A) the adoptively transferred recipients are compared to (B) 7 day secondary sera of the donor mice on Fd coated ELISA plates. Seven mice in each group; average values from all mice in a group ± S.E. are shown.
with 100 μg/ml normal mouse serum and 10 μg/ml rabbit anti-mouse Ig.
After washing, spleen cells from the donor mice were added to the dishes
at 7.5 x 10^7 cells in 5.0 ml. The dishes were incubated for 2 hr at
37°C and agitated every 30 min. Unbound cells were collected, and
adherent cells were removed by gentle scraping with a teflon policeman.
The cell recoveries are tabulated in Table 4. It can be clearly seen that
very poor separation was obtained by this technique, and the suggestion
can be made that in all probability, the cells denoted as "adherent cells"
are all macrophages, and the population of T-cells is a mixture of T- and
B-cells. With these results in mind, it is not surprising that the
results of the adoptive transfer depicted in Figure 30A, are at best
confusing. The low antibody production reflects the fact that only 5 x
10^6 immune T- and B-cells were transferred to each irradiated recipient
(the animals had been challenged 7 days after cell transfer).
II. Adoptive transfer II: Bl0.BR T- and B-cell transfer with the use
of nylon wool enrichment
For this second adoptive transfer, immune T- and B-cells were
obtained by nylon wool purification for T-cells and with α-Thy 1
monoclonal antibody (CBA Jij ascites, a generous gift from Dr. H.-S. Teh,
U.B.C.) for the enrichment of B-cells. Results of these two treatments
are shown in Table 5. Compared to the panning technique, more reasonable
results were obtained in terms of enrichment for immune T- and B-cells. 1
x 10^7 of each population were transferred to irradiated recipients. The
animals were challenged 7 days later. Unfortunately, the results of this
experiment (Figure 30B) are still extremely difficult to interpret as
Table IV  Panning technique: cell recovery for adoptive transfer I

<table>
<thead>
<tr>
<th>Donor a) immunized with</th>
<th>Original cell number (x 10^8)</th>
<th>Cell recovery</th>
<th>% recovery</th>
<th>% Adherent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>non-adherent (x 10^8)</td>
<td>adherent (x 10^7)</td>
<td></td>
</tr>
<tr>
<td>Fd</td>
<td>7.1</td>
<td>4.55</td>
<td>2.25</td>
<td>67.3</td>
</tr>
<tr>
<td>N</td>
<td>6.5</td>
<td>4.60</td>
<td>3.25</td>
<td>75.8</td>
</tr>
<tr>
<td>C</td>
<td>11.2</td>
<td>5.40</td>
<td>6.35</td>
<td>53.9</td>
</tr>
<tr>
<td>M</td>
<td>8.9</td>
<td>4.65</td>
<td>9.75</td>
<td>63.2</td>
</tr>
</tbody>
</table>

a) B10.BR mice were used.
Table V: T- and B- cell separation in adoptive transfer II

<table>
<thead>
<tr>
<th>Donor a) Immunized with</th>
<th>Spleen-Cell treatment</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-Thy + c b)</td>
<td>nylon wool c)</td>
<td>before (x 10^8)</td>
<td>after (x 10^8)</td>
<td>% recovery</td>
</tr>
<tr>
<td>Fd</td>
<td>2.80</td>
<td>1.4</td>
<td>50</td>
<td>4.20</td>
<td>1.0</td>
</tr>
<tr>
<td>N</td>
<td>3.23</td>
<td>2.0</td>
<td>62</td>
<td>4.38</td>
<td>1.8</td>
</tr>
<tr>
<td>C</td>
<td>3.03</td>
<td>2.0</td>
<td>66</td>
<td>4.80</td>
<td>1.6</td>
</tr>
<tr>
<td>M</td>
<td>3.43</td>
<td>2.4</td>
<td>70</td>
<td>4.50</td>
<td>1.8</td>
</tr>
<tr>
<td>N.I.</td>
<td>1.83</td>
<td>1.1</td>
<td>60</td>
<td>2.70</td>
<td>0.3</td>
</tr>
</tbody>
</table>

a) B10.BR mice primed and boosted with these antigens were rested 7 days before use.

b) α Thy 1.1 monoclonal antibody (CBA Jij ascites) was a generous gift from Dr. H.-S. Teh, U.B.C.

c) Nylon wool was prepared as described by Schwartz et al (116).
there were no non-immune B-cell or T-cell controls in each group, so the effect of mixing T- and B-cells from different immune donors could not be normalized. For this reason, it was decided that the next adoptive transfer would only employ transfer of immune T-cells.

III. Adoptive transfer III: B10.BR immune T-cell transfers and the effects of host irradiation

This experiment involved the transfer of immune T-cells and non-immune B-cells to either irradiated or intact recipients. Immune B10.BR mice were boosted with Fd or the enzyme fragments. Spleens and lymph nodes were collected from these mice 7 days after the boost, and passed over nylon wool columns (cell recoveries were between 19 and 38%). A non-immune B-cell enriched population was obtained with anti-Thy 1 + C treatment (66.5% cell recovery) of B10.BR spleen cells. $1 \times 10^7$ immune T cells were transferred to all recipients, and $1 \times 10^7$ non-immune B-cells were transferred to irradiated recipients only. The animals were challenged with Fd the day after the transfer. The results from this experiment are shown in Figure 31. It can be seen that transfer of immune T-cells to irradiated recipients results in a duplication of the response patterns of the donor animals, despite the fact that the recipients were all challenged with the native Fd molecule. At 14 days (the time when these bleeds were taken), a primary response is not measurable indicating that the magnitude of the responses were acquired by the transfer of immune T-cells.

At this point, it can be seen that "N"-immune T-cells, as compared to other sources of T-cells are the poorest mediators of immunity; no
comment can be made as to the nature of this low response i.e. lack of help or presence of suppression. Another interesting aspect of this experiment points out the necessity for sublethal irradiation of the recipients: intact recipients of syngeneic immune T-cells \((1 \times 10^7\) cells per animal) produce a substantially lower anti-Fd response, with no observable increase in anti-Fd antibody production with "N"-, "C"- and "M"-immune T-cells over transfer of non-immune T-cells. Only T-cells from Fd-immune animals could transfer increased immunity. Also, the primary response upon transfer of non-immune T-cells into intact animals is much higher than in the irradiated ones. Evidence for "help" from transferred cells could only be satisfactorily produced when four-fold higher immune T-cell numbers were transferred. These results are in agreement with the notion that mechanisms are present in intact animals which suppress the activity of the majority of transferred syngeneic cells. This suppressive mechanism is radiation-sensitive and most potent against lower cell numbers.

IV. Adoptive transfer IV: transfers of mixtures of C57BL/10 immune T-cell

This last adoptive transfer experiment was done in C57BL/10 mice as these mice have a very large antibody response to "C", as compared to Fd, and make no antibody by "N" and "M" immunization. These mice were primed, rested for 30 days, boosted, bled 7 days later (serum results shown in Figure 32B) and rested 45 days, sacrificed and used as donors of immune T-cells. Nylon wool purification of immune spleens provided an enriched T-cell population and anti-Thy-1 + C' treatment of non-immune spleens
provided the enriched B-cells. 92.6% of the nylon wool eluted cells were susceptible to anti-Thy-1 + C' lysis as determined by trypan blue exclusion, while with a second anti-Thy C' treatment, no additional cells were killed in the B-enriched population. Equal numbers of T- and B-cells were transferred to all mice; all mice received a total of $2 \times 10^7$ T-cells. The first group of irradiated recipients received $1 \times 10^7$ immune and $1 \times 10^7$ non-immune T-cells while the second group got $2 \times 10^7$ immune T-cells. Animals in the third group received mixtures of $1 \times 10^7$ immune cells from each of two different donors. The mice were challenged with Fd 2 days later.

From Figure 32A, it can be seen that transfer of $1 \times 10^7$ immune T-cells results in less clear results as compared to the transfer of $2 \times 10^7$ immune cells which perfectly mirror the response of the donor mice. Although the reason for this is not apparent, it may be proposed that at the relatively low dose of irradiation (450R), transfer of low cell numbers is adversely affected by radioresistant recipient cells yielding a similar situation to the one observed in Figure 30. Nevertheless, the following observations can be made concerning the data: the general effect of mixing Fd and fragment-immune T-cells is an additive one with very little evidence for the induction of cells by "N", which could suppress Fd-primed T-cells (the cells providing help in the response). The fairly low response of the "Fd + M" group may warrant further study as it is lower than the $1 \times 10^7$ Fd response. It therefore seems that, whatever the nature of the "suppressive" effect due to the N-determinant, the "N"-primed cells are not capable of shifting the balance between help
and suppression of Fd-immune T-cells in an adoptive transfer system.

V. Kinetics of the response

Figures 33 and 34 describe the kinetics of the response to Fd in the adoptive transfer recipients. In the B10.BR high responder mice (Figure 33), clear differences are observed at 7 days post challenge, with increasing responses with time. In C57BL/10, intermediate responders, where twice as many T-cells were transferred relative to the B10.BR assay, no response is observed at 7 days (Figure 34) while a good response is observed at 14 days. "N" and "M" immune cell transfer does not induce an adoptive (tertiary) response at any time (up to 4 weeks after challenge). It therefore seems that both the kinetics and cell numbers transferred reflect the relative frequencies of Fd-specific T-cells in B10.BR and C57BL/10 mice.

VI. Conclusion

The following definitive statements may be made. First, the recipients of syngeneic immune cells must be irradiated sublethally in order to observe any effects. Second, transfer of immune populations of spleen cells enriched for T-cells by nylon wool mimic the response in anti-Fd production of the donor animals (i.e. magnitude control is transferred by T-cells). No substantial comment can be made as to the generation of suppressor cells by "N" immunization, at any rate there is no generation of suppressor cells which can override a simultaneous transfer of helper cells from Fd-immune or "C"-immune animals. The antibody responses of the recipient mice appear between 7 and 14 days (depending on the strain, and possibly on the length of time the donor
animals were rested after the boost), and while the magnitude of the response increases slightly with time, the relative magnitudes of the responses between groups are constant.
Figure 33. Kinetics of the anti-Fd response of adoptively transferred B10.BR mice. Mice described in figure 31 were tested 7, 12 and 16 days on ELISA (coated with Fd at 1 μg/ml).
Figure 34. Kinetics of the anti-Fd response of adoptively transferred C57BL/10 mice. Mice described in figure 32 were tested 7, 14, 21 and 28 days on ELISA (coated with Fd at 1 μg/ml). 0—0 "C", •—• Fd, △—△ "N", △—△ "M", and ■—■ CFA.
Chapter 7. Discussion

Ferredoxin, a protein isolated from the anaerobe *Clostridium pasteurianum*, has been selected as an ideal probe for a number of immunoregulatory studies in this laboratory. The main goals of this thesis were to establish the nature of the murine immunogenetic control of the response to Fd, as well as to define the immunoregulatory functions of each antigenic determinant. The final objective was to assess the nature of non-responsiveness.

In Chapter one, a historical overview of the major antigenic systems was presented along with the previous studies in the Fd system. The purpose of this review was to highlight the properties of Fd as a good immunological tool: Fd, a non-mammalian protein, with no known autologous counterpart, minimizes autoimmune phenomena. Fd is also very small, at the lower limit of antigen immunogenicity at the B-cell level; it has two well defined non cross-reactive antigenic determinants which are not affected by structural manipulations to the rest of the molecule (i.e. responses to native and denatured forms of Fd are totally cross-reactive). Finally, Fd may be easily manipulated to yield "unideterminant" molecules.

The third chapter of this thesis provided evidence that the immune response to Fd was in fact under genetic control, mapping to the K-I/A subregion of H-2 with no apparent involvement of non-H-2 genes. The
fourth chapter explored the genetics of the response at the one
determinant level, with the basic conclusion that K/I-A controls not only
the magnitude of the antibody response, but its specificity as well. The
difference between intermediate and high antibody responses are due to a
"C"-specific increase observed in all high responder strains.

Chapters five and six explored the nature of the control at the
determinant level. Immunization with "unideterminant" molecules provided
clear-cut evidence that the N-determinant is a strong immunoregulatory
element of Fd, inducing both "suppressive" and proliferative functions,
while the C-determinant contains regions of high affinity for end-products
of the immune response: antibody and proliferating T-cells. The
phenomenon of non-responsiveness was also approached from a few
directions, yet only tentative proposals can be put forward at this time.

In this chapter, the results of this thesis will be discussed in
relation to some observations made in the literature concerning selected
systems. As certain phenomena are at variance with previous observations,
such as the assessment of proliferative responses, these will be analyzed
more closely. Finally, an attempt will be made to assimilate the data
into a model of immunoregulation of the response to Fd.

I. Genetics of the Response

In 1977, when the project was initiated, the current model of the H-2
complex contained the following subregions. K A B J E C S G D (260). It
was of interest to determine whether the response to Fd was under genetic
control, and to which subregion this control could be mapped. Through the
use of recombinant strains on the B10 background, the response to Fd was
localized to the area comprising K and I-A [by the response of B10.A(4R) mice, Figure 7].

From Table 1, where the immune responses to a panel of antigens are described, it can be readily seen that the majority have at least one gene mapping to I-A. Thus, the results with Fd do not stand out, excepting the fact that Fd is one of the few antigens with no demonstrable non-H-2 control. In retrospect, it is also not surprising to find that the response to Fd maps to the K/I-A subregion, as the recent reassessment of the H-2 complex by Klein and associates (14) now suggests the following genes or gene products: \( K_A A E E SDL \). The order of the underlined products is undetermined and localized within the original I-A subregion, with \( E_a \) localized within I-E.

Current sequencing studies by Hood and co-workers (261) provide physical evidence for the above proposal. They found that the majority of recombinant strains describing the I-B and I-J subregions were a result of recombination events within a small 1-3.5 kilobase segment, a segment too small to code for two separate gene products. The evidence for I-C was so weak, that this region is now left out of the current map. McDevitt (262) proposed that sufficient diversity could be generated through combinatorial interactions between the \( \alpha \) and \( \beta \) subunits (32K and 28K, respectively of I-A and I-E), to account for the regulation of all antigenic determinants (assuming that each Ia molecule can interact with foreign antigens via multiple discreet sites). Klein et al (14) went on to propose that the above gene products are sufficient to account for all
the manifestations of immunoregulation: mixed lymphocyte reactions, control of the immune response, control of T-B collaboration, and the restriction of T-cell specificity.

These modifications of the H-2 may in part explain the previous difficulty in the assignment of Ir gene control to a number of antigens. In fact, to date the presence of the I-B subregion is avidly contested. For example, Nadler et al. (232) provide evidence that the response to staphylococcal nuclease definitively maps to I-B and is not a result of complementation between I-A and I-E as would be suggested by proponents of the Klein model. Reconciliation of this discrepancy must await rigorous experiments, including extensive sequencing studies.

A source of difficulties in genetic mapping of immune responses is the multideterminant nature of most antigens used. In elegant studies with well characterized antigens such as insulin (146,148), myoglobin (84) and staphylococcal nuclease (224,235), it became clear that there exists individual Ir gene control for each antigenic determinant on a complex antigen. One can see that through immunodominance or immunoregulatory control, the net genetic control of the response can be assessed, providing that the antigen has a small number of antigenic determinants.

Therefore, in actuality, only mapping to one of two regions needs to be accomplished; the task remains in finding whether any genes in I-E control the response. In doing so, it must be kept in mind that the b and s haplotypes lack the $E_\alpha$ gene product (263), such that the interacting surface structure $E_\alpha: E_\beta$ (or $A_e$) is not present in these strains [although cytoplasmic $E_\beta$ is present (264)]. Another complicating factor
must be kept in mind: recent studies involving the use of monoclonal antibodies have demonstrated that alloantigenic determinants are shared by I-A and I-E (indicating a gene duplication event) (265) which may in some cases result in $A_\alpha^E_\alpha$ or $A_\beta^E_\beta$ complementation as well, in a gene dose related fashion.

With Fd, all available recombinant strains, except B10.HTT and A.SW, conformed with the mapping of the response to K/I-A. In the case of A.SW, analysis is difficult as A.SW (providing the H-2 to B10.S) make antibody, and A/J mice (providing the non H-2 gene) also respond. With B10.HTT one of the suggestions would be complementation, yet no discernible pattern can be found to explain this low response. This result at this time can only be attributed to a mutation in this strain.

A stand-out feature of the Fd response is that non-H-2 effects were not found. All major antigenic systems that have been studied to date have found some effects by genes outside of H-2. For example, the A background has been implicated in promoting a positive effect in the control over the magnitude of the response to a large number of antigens: HEL (174), myoglobin (87), GAT (30), staphylococcal nuclease (228) and (T-G-A-Gly)n (60). This effect is evident at different stages of the immune response; for example, with HEL, the non-H-2 effect in A/J mice is only evident in the secondary response where 25-fold higher amounts of antibody are produced than by B10.A mice; this effect was not seen during the primary response (175). This recurrence in many antigenic systems of high antibody levels with the A background makes the interpretation of the discrepancy with A.SW in the Fd system even more difficult: A.SW make no
anti-Fd antibody as compared to SJL and B10.S which are intermediate antibody producers, yet A/J mice do respond.

It can be concluded that fairly clear mapping of the Fd response was possible as Fd has only two antigenic determinants. The control is as follows: $A^k A^k$ dictates a high response to Fd (with high specificity for the C-determinant, $A^b B^b$ and $A^s A^s$ dictate an equivalent response to both determinants, while $A^d A^d$ indicates non-responsiveness to Fd. In the case of $A^d A^d$ the question remains as to whether the lack of response is due to suppression or to a lack of interaction between the Ia antigens and the determinants of Fd.

The final comment concerning genetic control of the immune response deals with the inheritance of the response genes by $F_1$ mice. Despite the lack of a formal $F_2$ analysis, inheritance of Fd response genes follows the gene dose response phenomenon at the antibody level of codominance as calculated for most systems by Ebringer et al. (266) and dominance at the proliferative level. Therefore, expression of $A^k A^k$ and $A^s A^s$ is codominant in $(H-2^k \times H-2^s)F_1$ and results in an antibody response of magnitude intermediate between the two.

**II. Determinant Specificity: T- Versus B-cell Repertoires**

As one of the most important features of the immune system is its antigenic specificity, a major interest with protein antigens is the definition of stimulatory sequences, or antigenic determinants. Although a number of innovative approaches have been followed, a philosophical question needs first to be presented: are antigenic determinants molecular entities that are recognized by the immune system due to their
variance from self, or because they assume a physical "shape" which, regardless of identity with self, is immunogenic? In the literature, the two possibilities are encountered by researchers studying mammalian protein antigens which have many species variants. For example, in a study of 15 species variants of myoglobin, where antisera to each were scored for cross-reactivity, it was found that the same or similar sequences were recognized by the antisera irrespective of the immunized species, and that antisera to various myoglobins cross-reacted with the autologous molecule (198,213). Similar cross-reactivity in antibody responses was observed with the antigen serum albumin, with the conclusion that the antigenic sites are independent of the immunized species (95). The evidence for this supposition lies in the fact that autoimmune T-proliferative (210) and antibody (197) responses can be readily measured (myoglobin system). Atassi also demonstrated that some of the antigenic determinants of cytochrome c are dependent on conformation and not sequence differences, and that this region (residues 42-50) is responsible for autoreactivity (126).

Therefore, based on a number of systems under study, Atassi and co-workers propose that immunogenicity is dependent on the spatial arrangement of the antigenic determinant irrespective of sequence differences from the autologous protein. Actually, this interpretation is applicable in the study of autoimmune phenomena. For example, in the experimental murine autoimmune thyroiditis systems, it has been found that H-2 linked genes were responsible for both autoimmune and cross-reactive T-lymphocyte proliferation (267). The antibody response was also mapped
to the same I-A subregion with further proposed involvement of H-2D genes (268).

Most other researchers have adopted views either slightly at odds with the ones described above, or totally contradictory to them. In the first case, a study of single amino acid substitutions of cytochrome c by Urbanski and Margoliash (112) support a proposal that varies only slightly from Atassi's: topographic determinants are recognized by the immune system, but these only arise as a result of single amino acid changes (conservative) between the immunizing antigen and the host counterpart. Most other investigators however, assign the fine specificity of immune responses solely to areas of the antigen that are at variance from self counterparts.

Examples of the above approach are seen again in the cytochrome c system at the T-cell (proliferative) level (120). Residues 100, 103 and 104 were found to be critical in the immunogenicity of the variants as compared to pigeon cytochrome c. Unfortunately, comparisons to Atassi's findings are difficult as he dealt with beef and rabbit cytochromes (126). Nevertheless, the discrepancy exists in the interpretation of the basis of immunogenicity of a highly conserved system.

With lysozyme, residue 3 has proven to be a site with high correlation to immunogenicity through the use of a large array of species variants (176,177). However, Atassi again, through vigorous immunochemical approaches had described different antigenic determinants based on residues that are usually, but not necessarily conserved between species variants (171).
An interesting finding in the myoglobin system by Berzofsky et al. (211) contradicts Atassi's finding described above (198). In the study of two myoglobins, equine and sperm whale, it was found that totally different genes were responsible for the control of these antigen, the response to sperm whale myoglobin mapping to I-A and I-C, while the response to the equine variant was found to be controlled by complementing genes in I-A and H-2D. The suggestion that can be put forward in this case is that, as different genes are responsible for the response, shared antigenic determinants cannot be implicated despite minimal variance between the two molecules.

Whatever the correct definition of antigenic determinants will be, a compromise between the two views can be proposed to satisfy most observations. As this dichotomy between views exists, studies with mammalian proteins suffer due to the antigenic complexity and possible autoreactive components of the response. Advantages gained with the use of non-mammalian proteins such as staphylococcal nuclease and ferredoxin become clear. However, staphylococcal nuclease, which is almost 3 times as large as Fd, has a complex pattern of genetic control as compared to Fd. Also, its antigenic determinants have not been defined beyond reactivity to large CNBr peptides.

Whatever the nature of the antigenic determinants, agreement with respect to the nature of B- versus T-cell repertoires is more universal. From early studies with native molecules and their peptide fragments, a discrepancy between T- and B-cell repertoires was found. For example, after the advent of reliable proliferation assays where T-cell specificity
could be analyzed, it was observed that peptides no longer binding antibody to the native molecule, could stimulate specific T-cell proliferation. For example, in the staphylococcal nuclease system, CNBr fragments devoid of the tertiary configuration mandatory for antibody recognition could stimulate T-cell responses leading to the postulate, now widely accepted, that T-cells recognize antigenic determinants similar in both the native and denatured form of the protein (229).

The most elegant studies analyzing the selection of determinants by the immune system can be attributed to Rosenthal and co-workers utilizing the insulin system to describe the macrophage as the cell which physically "selects" determinants and presents them to T-cells in the context of self Ia antigens (146, and reviewed in 269). Since that time, the majority of systems dealing with complex protein antigens, having described the variation between T- and B-cell repertoires, attribute the difference to the fact that antibody interacts with antigen in "soluble" form, whereas T-cells interact with antigen in the context of APC and Ia. The fact that total cross-reactivity between native and denatured forms of the antigen exists, for example in the BSA and OVA systems, with no concomitant cross-reactivity at the B-cell level (92), the prediction can be made that the APC reduces both antigenic forms to a common representation to the T-cell.

With the ferredoxin system, there is a good chance that complications due to the configurational state of the molecule do not exist. It can be proposed that Fd, which is only 5600 M.W., is too small to be extensively degraded by the APC, such that the form recognized by antibody bears close
resemblance to the one presented by the APC to T-cells, (K.C. Lee, personal communications). For this reason, the expectation has been maintained that T- and B-cell repertoires for Fd reactivity are similar, if not identical.

In view of the results presented in this thesis, the absolute identity between T-cell and B-cell specific determinants has not been proven. In fact, limited evidence exists suggesting that more T-cell-reactive "areas" exist than B-cell "areas" on the Fd molecule. Results from a few of the adoptive transfer experiments suggest that T-cell priming may occur in the absence of antibody production in the donor animal. In fact, immunization with "M", which induces no antibody formation and does not react with anti-Fd antibody, still primes for and stimulates considerable T-cell proliferation in vitro. This reactivity still needs to be localized: it may be directed either to the stubs of the determinants (residues 4, 5, 6, 7, 51, 52 and 53) or to a third T-cell determinant which would be localized within residues 8 to 50 of Fd.

A general statement may be made at this time concerning the antigenic determinants of Fd: within the limits of the analysis, Fd has two determinants at the B-cell level, and at least two at the T-cell level. As antibody binds native and modified Fd equally well, the conformation of the determinants is not inflexible at the B-cell level. A presumed lack of extensive APC processing predicts that the Fd-specific T- and B-cell repertoires are highly related, but in all probability not identical. This property of Fd will make eventual model building more feasible. As T- and B-cell antigenic determinants can be perceived to be
similar, idiotypic networks can be assimilated into classical hapten-carrier models of immune responses, where determinants in a simple molecule assume either hapten or carrier properties. This topic will be pursued in the final section following the assessment of the proliferative assay as a measure of immunity.

III. The Proliferative Assay: Assessment of Immunity

Prior to 1975, the majority of studies of T-cell functions were performed in rabbits and guinea pigs as adequate culture conditions were unavailable for murine *in vitro* antigen specific proliferation assays. After Click *et al.* (258) described a complete culture medium suitable for the support of murine cell cultures, Schwartz *et al.* (116) devised a reliable method for studying T-cell phenomena in mice using peritoneal exudate T-cells (PETLES). With the use of this assay, the authors subsequently compared the proliferative and antibody responses of various mouse strains to a panel of antigens (270). It was found that not only genetically related differences originally observed in antibody production (high, low and non-responders) could be duplicated by the proliferative assay, but that in two cases, proliferation was mapped to I-A as was the antibody response. The suggestion was made at the time, that immune response genes were not limited to B-cell expression, but were also found in association with T-cell (and/or macrophage functions), and that the same response gene was actually involved.

With the availability of the PETLES assay, the nature of the proliferative response was analyzed. It was soon recognized that an antigen recognition phase preceded the proliferative phase in *in vitro*
T-cell proliferation (271). In a more in depth study, it was calculated that three cells were involved in the T-proliferative response: two T-cells, one antigen specific and the other one not, and an antigen presenting cell (272). For GAT it was estimated that one in $1.3 \times 10^6$ cells were antigen specific proliferating cells, while the bulk of proliferating cells were non-specific (or unprimed) cells. Further studies attempted to characterize the type and function of the proliferating cell.

Schrier et al. (273) demonstrated that antigen induced proliferation was dependent on a Ly $1^+$ cell which provided a source of T-cells supplying help in an antibody assay. Although no evidence was presented that the proliferating cells were identical, the argument was that, since serial propagation (cloning) produced cells capable of helping hapten-carrier responses, the cells were the same. Furthermore, in a 1981 publication, Walker et al. (274) proposed, with the use of splenic cells [as opposed to PETLES or lymph node cells as used by Corradin et al. (117)], that with their assay, proliferative responses could be correlated to B-cell responses as the same cell source was used for both assays. Their results were in agreement with previous studies in that the proliferating cell was found to be Ly $1^+23^-$ and presumed to reflect an expression of T-cell activity.

On the basis of the above results, the majority of investigators have employed the proliferative assay as a means of delineating the genetics of the response of a number of antigens. However, on the basis of the results obtained in the Fd system, as described in previous
chapters of this thesis, a re-examination of the correlation between T-cell proliferation and T-cell help in antibody responses must be undertaken. The instances of dichotomy between proliferative and antibody responses are enumerated:

1. In the comparison of the proliferative response (Figure 2) and antibody production (Figure 4), the lack of correlation between the two assays is already apparent: H-2^k and H-2^s mice undergo a proliferative response, whereas H-2^b and H-2^d do not. H-2^k mice produce high levels of antibody, H-2^b and H-2^s intermediate and H-2^d make none. Therefore, although absolute responders, H-2^k and non-responders, H-2^d, correlate, there is a lack of correlation for the intermediate antibody producers, C57BL/10 not proliferating while the proliferation by SJL and B10.S equals that of high responders. Therefore, antibody was produced by C57BL/10 mice in the absence of proliferation.

2. In a further study with A.SW, which on the basis of H-2 genetics were predicted to be proliferators and intermediate antibody producers, an anomaly was found when no antibody production was observed despite a good proliferative response. Therefore, the opposite situation was found from the one above where a good antibody response was observed without a matching proliferative response.

3. With the use of "unideterminant" (at the antibody level) fragments of Fd, the split between the two measures of immunity was evident under a number of experimental conditions. Figures 25, 26 and 28 describe magnitudes of anti-Fd antibody in fragment immune mice. The following observations may be made:
a. With B10.BR mice, immunization with "N" induces a Fd-specific proliferation equalling that of native-Fd primed mice, yet there is virtually no Fd-specific antibody found in the sera of these mice.

b. Immunization of B10.BR mice with "C" produced the converse situation to the one commented on above such that poor Fd-specific proliferation was observed while a very high anti-Fd antibody response was induced.

c. C57BL/10 mice were found to be incapable of proliferating not only to Fd, but to all the fragments as well, despite the fact that an intermediate antibody response was seen with Fd immunization and a high response to immunization with "C".

d. B10.D2 mice were also uniformly non-proliferators yet immunization with "C" of these Fd non-responders induced an antibody response unparalleled by a proliferative response.

e. A.SW mice which proliferate to Fd when immunized with Fd yet make no anti-Fd antibody, do not proliferate to Fd following immunization with "C" which induces an antibody response.

It is therefore clear that in a well-defined system such as Fd, the assessment of immunity varies depending on the assay used. Originally, an explanation was put forward that antibody, being a measure of the end product of an immune response, does not necessarily reflect immunity, as the regulation may break down after the theoretically earlier manifestation of immunity, T-cell proliferation. This, however, can no
longer be supported as a number of cases exist where an antibody response is seen in the absence of concomitant T-cell proliferation. Therefore, I propose that the proliferative assay may not be used to assess the presence or absence of T-cell help and that this assay is a reflection of a single characteristic of the response, proliferation, with no apparent functional role for the proliferating cell. I believe that the previous conclusions on the nature of the proliferative response stem from the fact that most studies were performed with large multideterminant antigens, and not with molecules as well defined as Fd.

Careful studies undertaken recently with better defined systems have produced evidence supportive of observations made in the Fd system. In an elegant study (275,276) on the nature of proliferating cells to both KLH and apoferritin, it was found that although the presence of helper T-cells could be assessed in cultures with both antigens, only KLH induced high levels of proliferation, levels equalling proliferation to polyclonal activators. The high KLH-specific proliferation was attributed to trans-stimulation of non-antigen-specific cells, which proliferated. In fact, it was found that KLH-specific cells did not need to proliferate in order to induce trans-stimulation. The results of this study could, therefore account for the previous observations by Schrier (273), where help and proliferation were equated.

The distinction between proliferation and help was also made in the β-galactosidase system where eleven CNBr fragments of GZ were compared in both facilities (134). Although 9 out of 11 peptides primed for a degree of proliferation, only two were instrumental in T-helper functions. In
the lysozyme system, CNBr fragments were also used to compare T-cell and B-cell repertoires (182). It was found that although the N-C determinant was responsible for the majority of antibody activity, the T-cell proliferation response was highest with the $L_{\text{II}}$ fragment. This was used as evidence for a hapten-carrier model where $L_{\text{II}}$ provided the T-cell directed (carrier) stimulus, while the N-C fragment was the B-cell oriented (haptenic) moiety. Although similar conclusions may be reached in the Fd system ("C" reflecting the hapten and "N" the carrier), there is still a lack of explanation for the inconsistency of C57BL/10 and A.SW responses. What one has to invoke is the involvement of suppressor determinants in the regulation of proliferative and antibody responses.

The GAT system has historically been the model for studying suppression. Araneo et al. (42) have demonstrated that non-responder T-cells do not proliferate in vitro as a result of priming with GAT, but do with GAT-MBSA, which concomitantly induces antibody formation, the suggestion being made that lack of proliferation reflects active suppression. This phenomenon is however not universal, as other systems can demonstrate T-cell proliferation by determinants encoding suppressive functions. In the insulin system studied with guinea pigs, it was observed that the regions of the molecule that are involved in the induction of anti-hapten suppression (at the antibody level) are the same as those involved in inducing proliferative responses (154). These investigators found that carrier-specific suppression only affected anti-hapten antibody responses while the anti-carrier response was unaffected. These results indicate that the same sequence in insulin is
responsible for induction of proliferation, help and suppression. The previously mentioned studies with GZ (134) corroborate these findings as the CNBr fragment, CB-2, was found not only responsible for proliferation and help, but suppression. With regards to the Fd system, the N-determinant was implicated with suppression, yet it is also the determinant which induces a proliferative response. Therefore, our results with Fd do not correlate with those obtained with GAT, but are not at odds with the GZ and insulin data.

IV. In Anticipation of Model Building

It is always extremely satisfying when all of the information obtained for an immune system can be incorporated into a model. The best available example, by far, is the lysozyme system, where not only immunogenetics and determinant specificity are known, but extensive knowledge of the idiootypic control is available. A model of the lysozyme system initiated in early reviews by Sercarz and co-workers (170,176) has been presented elegantly in a later publication (277), where idiotypic control was shown to complement hapten-carrier regulation. It is the hope of the investigators concerned with the Fd system to eventually produce a model of cellular interactions accounting for the immune response to Fd. Although at this time, the data available is insufficient for model building, a few theories will be presented that may indicate what direction some of the future investigators may take.

The current postulate of immune regulation revolves around the fact that genetic restriction through Ia molecules determines the state of responsiveness of an H-2 haplotype, to a given determinant. It has been
shown in a number of systems that blockage of T-proliferation and antibody production could be achieved by anti-Ia antisera or monoclonal antibodies (53,220,278,279). The conclusion obtained is that as a result of antigen processing, antigenic determinants are presented on APC's in the context of Ia molecules. This complex is subsequently recognized by subsets of T-cells. This recognition is inhibitable by antisera at least to the public specificities of the Ia antigen.

As elegantly communicated in a letter to Nature, Schwartz (280) presents the only unsolved problem regarding Ia dependent regulation of responsiveness, and more specifically, the nature of non-responsiveness: on one hand, proponents of the determinant selection model (269) argue that non-responsiveness is a result of the failure of non-responder Ia molecules to bind the antigenic determinant in the APC, while the opposing view proposes that the non-responsiveness is due to a lesion in the T-cell repertoire for a given combination of Ia and antigen which simulates self (281). This question will hopefully be addressed and answered in the Fd system by future investigators.

It is possible, however, that clues to Ia specificity involvement may already be present. In the Fd response, B10.BR mice are high responders, C57BL/10 and B10.S are intermediate responders and B10.D2, non-responders. Each of these strains has individual and shared, serologically defined determinants of I-A, the gene(s) found to code for the Ia restriction element of this response. Table VI lists an arbitrary grouping of the serologically defined Ia specificities according to haplotypes and phenotypes of the Fd response.
<table>
<thead>
<tr>
<th>Group</th>
<th>Haplotype</th>
<th>serologically defined Ia specificities (*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>d</td>
<td>Ia.6, Ia.11, Ia.16, and Ia.23</td>
</tr>
<tr>
<td>2</td>
<td>s, b, d</td>
<td>Ia.30</td>
</tr>
<tr>
<td>3(A)</td>
<td>k, b,</td>
<td>Ia.3</td>
</tr>
<tr>
<td>(B)</td>
<td>k.s</td>
<td>Ia.17, Ia.18 and Ia.33</td>
</tr>
<tr>
<td>4</td>
<td>k</td>
<td>Ia.1, Ia.2, Ia.18, Ia.22, Ia.25, Ia.26, Ia.28, Ia.29, Ia.31 and Ia.32</td>
</tr>
<tr>
<td>5</td>
<td>k, b, d</td>
<td>Ia.15</td>
</tr>
</tbody>
</table>

\(\text{(*) Data obtained from Klein et al (282). The specificities unique to each allele or haplotype of I-A are listed, as well as those specificities shared by two or more haplotypes.}\)
Group 1 represents specificities unique to H-2^{d} non-responder mice. Interaction of Fd determinants in a manner proposed by the clonal deletion model (281) can lead to non-responsiveness through cross-reactivity with self. As immunization with "C" of H-2^{d} mice yields responsiveness, it could be proposed that the Ia specificity responsible for non-responsiveness in H-2^{d} possibly interacts with the N-terminal (tripeptide). Group 2 contains a single specificity, Ia.30, which is common to b, s and d haplotype animals. It could be that this is again an autoreactive specificity in the Fd system. As the difference between intermediate and high responder status reflects an increase in C-reactive antibody, it could be proposed that interaction of Ia.30 with the C-determinant is responsible for C-specific clonal deletion of T-cell recognition. Group 3 contains two types of specificities, those shared by the high responder, haplotype k, and the intermediate responders, haplotypes b (3A) and s (3B). This would constitute the first example of positive selection of determinant specificity by Ia antigen. For the moment, let Ia.3 represent "N" specific presentation for k and b T-cells, and Ia.17, Ia.18 and Ia.33 show specificity for "N" and "C" reactivity of k and s mice. Similarly, group 4 specificities, all possessed by k mice, represent some "N" and mostly "C" reactivity. Lastly, group 5, contains one specificity, Ia.15, which selects for "C" responsiveness of k, b and d mice. This specificity would account for a "C" determinant response with immunization with the "C" fragment.
Admittedly, the above proposal is strictly theoretical and can only be substantiated with monoclonal antibodies to these I-A specificities and selected mutant strains to correlate to the serological approach. Nevertheless, such a regulatory process by Ia molecules accounts not only for the response status but the specificity of the response as well.

The following is a summary of data obtained to date in the Fd system, which at some date (when sufficient information will be available) can be successfully incorporated into an all-encompassing model of the Fd response.

1. Fd, a 55 amino acid electron transport protein isolated from *C. pasteurianum* is an antigen with no known mammalian counterpart and limited cross-reactivity with any mammalian molecule. However, early unpublished studies in this laboratory have found limited cross-reactivity with the carboxy terminus of cytochrome *c* at the antibody level. This may be in part due to the fact that cytochrome *c* is also found in procaryotes such as *Desulfovibrio vulgaris* (240). In that case, 20% identity was found with Fd (239); as this difference has statistically proven to be insignificant in terms of common ancestry reflecting no similarity in crystal structure; the cross-reactivity must be due to the similar C-terminal sequence.

2. Modification of cysteine residues of Fd (it has eight) yielded no major interference with the immunogenicity of Fd (1). This fact suggested that non-cysteine containing sequences represent the determinants of Fd. Cysteine-rich sequences are limited to
central portion of Fd, while both the amino and carboxytermini are cysteine-free.

3. Synthesis of NH$_2$- (4) and COOH- (3) terminal sequences has defined two antigenic determinants of Fd.

4. Two peptides, representing these two determinants, were found to account for the non-cross-reactive binding of all rabbit anti-Fd antibodies (4,5).

5. A number of extensive studies reviewed elsewhere (9), indicated that the N-determinant is involved in the majority of immunoregulatory functions while the C-determinant has higher affinity for B-cell functions.

6. Fd is immunogenic in rabbits, guinea pigs and mice, the same antigenic determinants are involved.

7. The response to Fd is K/I-A controlled with no apparent involvement of non-H-2 genes.

8. The relative proportion of N- and C-specific antibodies is also controlled at K/I-A: I-A$^k$ mice make 80-90% C-specific antibody, while I-A$^s$ and I-A$^b$ mice make a 50:50 response to the two determinants.

9. The absolute amount of N-specific antibody is the same in k, b, and s haplotypes.

10. Preliminary experiments with H-2$^d$ animals, whose T-cells were pretreated with rabbit anti-Fd-2 antiserum plus complement (Fd-2, or 17-3, is a C-specific monoclonal antibody with very limited serum representation) indicate that these converted
non-responders produce an anti-Fd response of similar specificity as intermediate responders (244). An anti-idiotype produced to an N-specific antibody was also found to be involved in immunoregulation as pre-treatment of B10.BR T-cells yielded marked increases in anti-Fd responses (245,246). To date, treatment with both anti-Fd-1 and anti-Fd-2 has not upset the ratio of determinant specificity. In view of the proposal of determinant reactivity due to clonal deletion and Ia serological specificities, it is not surprising that this ratio does not change. It is obvious that idiotypic control of the immune response is at this time separate from the genetic control of Ia, which determines responsiveness status. More intensive studies will be necessary to provide the link between idiotypic and I-A control. The other aspect, hapten-carrier regulation, plays a role in both these controls and it may provide the link between the two aspects of immunoregulation.

11. The most informative experiments have been concerned with the perturbation of the Fd-specific immune system with "unideterminant" fragments of Fd. Confirmation was obtained at the antibody level, that only two antigenic determinants exist in Fd ("M" neither binds nor induces anti-Fd antibody), a hapten-carrier relationship within Fd can be established, similar to the one in early glucagon studies (135). "N" is clearly an immunoregulatory determinant inducing both carrier helper functions to the C-determinant and possibly suppressor
functions to the N- and C-determinants as well as priming for a proliferating T-cell. "C" has highest affinity for the end products of an immune response: antibody and primed T-cells that proliferate in vitro.

12. Studies covered in Appendices VI and VII of this thesis have dealt with the determination of the nature of non-responsiveness of H-2^d mice. However, both cyclophosphamide treatment of mice as well as immunization of Fd linked to an immunogenic conjugate was unsuccessful in describing active suppression as the reason for non-responsiveness.

13. Recent unpublished studies performed as an extension of this thesis have been also supportive of the lack of active suppression in non-responsiveness: the co-immunization experiment described in Figure 23 was repeated with new fragments with totally different results. It was found that immunization with N + Fd had an additive and not a suppressive effect. This result is considered more trustworthy as the fragments in the previous experiment were uncharacterized. An underestimate in concentration may result in a lowered response if one considers the sharp dose-response curve of Figure 1.
Although more information must be generated before a comprehensive model for the immunoregulation of the response to Fd can be constructed, a preliminary description of T- and B-cell determinants may be proposed:

A. At the antibody level. As the N-terminal tripeptide (site 1) and C-terminal dipeptide (site 2) are critical in the binding of any anti-Fd antibody (both heterogeneous serum antibody and monoclonal antibody), it may be proposed that these are the only two B-cell determinants (hence the definition of Fd as a bi-determinant molecule).

B. At the proliferative level. As only high responder B10.BR mice demonstrated any appreciable proliferative response to fragment immunization, it can thus be demonstrated that the site 1 sequence is responsible for the largest induction of proliferation. As intermediate responder C57BL/10 mice demonstrate a very weak proliferative response, it may be proposed that the site 1 determinant in these mice is weak (with respect to B10.BR), while it is absent in B10.D2 (non-responder) mice.

C. At the T-helper level. Three 'determinants' can be proposed at the T-cell level in order to satisfy the observations made to date in the Fd system:
1) Evidence for site 3: As immunization with "C" induces an antibody response in non-responder mice, and an increased response in intermediate and high responders (with respect to the response to the native molecule), the cleavage of the amino terminal tripeptide uncovers a sequence or configuration acceptable to a population of T-helper cells. These cells cannot recognize this configuration adequately in the native molecule, hence the non-responsiveness status of B10.D2. This T-helper cell provides help to the C-determinant-specific B-cell response.

2) Evidence for site 2: Site 2 is recognized to an equal extent by C57BL/10 and B10.BR but not by B10.D2 as the magnitude of the aN responses is identical in the responding strains.

3) Evidence for site 1: as for the proliferative response, this site is missing in non-responder mice as no C-specific response is measured in Fd-immunized mice. This site is very potent in high responders while poor in intermediate responders. This is reflected in the N:C ratios of anti-Fd sera of these strains: the net increase in magnitude of B10.BR over C57BL/10 is accounted by an increase in C-specific antibody (via hapten-carrier).

D. At the T-suppressor level. Although initial results with immunization with "C" suggested a suppressor determinant at site 1, the need for this assignment generally disappears as most data obtained with fragments satisfies the above criteria. Where suppressor determinants are still implicated are in the idiotypic studies (244,245,246) mentioned above. In order to clarify the interaction between Ia control of the determinant specific responses and idiotypic networks of control, more
information must be generated before a comprehensive model for the immunoregulation of the response to Fd can be constructed:

1. The quantitation of major idiotypes to both the "N" and "C" determinants (currently carried out by Doug Demetrick).

2. The confirmation of the presence of these idiotypes on major immunoregulatory cell subsets, with the subsequent characterization of these subsets.

3. Verification of the four group hypothesis represented earlier in Table VII with respect to Ia controlled responses. This task to be undertaken with a series of monoclonal antibodies to the Ia specificities predicted to be involved, as well as selected Ia mutant strains of mice.

4. Finally, the cloning of T-helper and suppressor cell lines will be needed as proof for the recognition elements for the (Ia + antigenic determinant) specificities.

In conclusion, I hope that the merits of the Fd system in supplying conclusive evidence for the study of immunoregulatory phenomena can be seen. It is a well-defined system that is likely to provide clear evidence for many immunological phenomena. This thesis has provided evidence that the murine anti-Fd response is controlled by the K/I-A subregion of H-2. This control is manifested by the action of three helper determinants in high responder mice yielding a predominantly C-determinant specific response. The intermediate status is a result of a much poorer recognition by T-helper cells of the NH₂-terminal determinant (site 1) while the non-responsiveness status reflects the lack
of recognition of both the NH$_2$-terminal (site 1) and the COOH-terminal (site 2) determinants. The selective cleavage by trypsin has uncovered the presence of a third determinant (situated most probably on the stub at the NH$_2$-terminal – although precise mapping has not been undertaken. This third determinant renders Fd-non-responder mice into responders without the necessary implication of suppressor cells.
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245. Weaver, M., Sikora, L., and Levy, J.G. 1982. Effects of anti-idiotypic sera (Ab-2) and monoclonal idiotypic antibody (Ab-1) in the immune response to a simple polypeptide antigen with only two immunologically active epitopes. Analysis of the response at the undeterminant level. In EMBO Workshop on Protein Conformation as Immunological Signal (Celada, F., Sercarz, E., Shumacher, V., eds.).
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Appendix I. Genetic information on mice used in mapping studies

Table VII - H-2 genetics of mice used in mapping studies (282)

<table>
<thead>
<tr>
<th>Strain</th>
<th>heavy chain allotype</th>
<th>H-2 haplotype</th>
<th>H-2 haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.A(2R)</td>
<td>b</td>
<td>h2 1)</td>
<td>k k k k k d d d d</td>
</tr>
<tr>
<td>B10.D2</td>
<td>b</td>
<td>d</td>
<td>d d d d d d d d</td>
</tr>
<tr>
<td>B10.A(4R)</td>
<td>b</td>
<td>h4 1)</td>
<td>k k b b b b b b b</td>
</tr>
<tr>
<td>B10.A(3R)</td>
<td>b</td>
<td>i3 1)</td>
<td>b b b k d d d d</td>
</tr>
<tr>
<td>B10.A(5R)</td>
<td>b</td>
<td>i5 1)</td>
<td>b b b k k d d d d</td>
</tr>
<tr>
<td>B10.BR</td>
<td>b</td>
<td>k</td>
<td>k k k k k k k k</td>
</tr>
<tr>
<td>B10.S</td>
<td>b</td>
<td>s 2)</td>
<td>s s s s s s s s s</td>
</tr>
<tr>
<td>B10.HTT</td>
<td>b</td>
<td>t3 3)</td>
<td>s s s s k k k k k</td>
</tr>
<tr>
<td>B10.S(9R)</td>
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<td>t4 4)</td>
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</tr>
<tr>
<td>C3H</td>
<td>j</td>
<td>k</td>
<td>k k k k k k k k</td>
</tr>
<tr>
<td>CBA</td>
<td>j</td>
<td>k</td>
<td>k k k k k k k k</td>
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<td>k</td>
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</tr>
<tr>
<td>C58</td>
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<td>k</td>
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<td>A.SWN</td>
<td>e</td>
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<td>SJL</td>
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</tr>
<tr>
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<td>c</td>
<td>d</td>
<td>d d d d d d d d</td>
</tr>
<tr>
<td>NZB</td>
<td>e</td>
<td>d</td>
<td>d d d d d d d d</td>
</tr>
</tbody>
</table>

1) These recombinants are a result of events between the A/WySn donor strain and C57BL/10, the inbred partner.

2) A recombinant between donor strain A.SW and the inbred partner C57BL/10.

3) Recombination between H-2-t1 a noninbred line (generated via recombination between A.AL and A.SW) and the inbred partner, C57BL/10.

4) A recombinant between A.SW and B10.A and then the inbred partner, C57BL/10.
Appendix II. Growth of Clostridium pasteurianum, isolation, purification and quantitation of ferredoxin

The freeze-dried culture was obtained from the American Tissue Typing Association, ATCC # -2-6013. Methods originally described by Mortenson (247) were employed, with a few modifications, in the production of ferredoxin from cultures of C. pasteurianum.

A) Growth of C. pasteurianum

Under aseptic conditions, the contents of the ampoule were resuspended in 1-2 ml sterile saline and transferred to 30 ml of glucose peptone beef infusion (GPBI) medium, freshly boiled to drive off $O_2$ and cooled and supplemented with sterile glucose to a final concentration of 2% (w/v). The culture was incubated for 18 hours at 30°C; 3 ml of this culture were subcultured into fresh GPBI tubes until good growth was observed by 18h (manifested by copious bubbling and the ground meat plug is pushed up on top of the broth). At this stage, the bacterial cultures is ready for 10-fold step-up subculture into a semi-synthetic culture medium, the 300 ml culture described below. The entire GPBI tube is transferred to the freshly boiled and cooled medium, supplemented with 2% glucose. The growth must again be optimal at 18 hours, manifested by a foam filling the 500 ml flask. Only when this stage is reached, can the next two, 10-fold step-up procedures to totally synthetic medium (3 l and 30 l) be made. Again both of these must be freshly cooled after boiling and maximal growth must be observed within 18 hours. The 30, 300 and
3000 ml cultures are incubated at 30°C, whereas the 30 l one is usually kept at room temperature. When the 30 l culture has peaked, it must be harvested immediately by continuous flow centrifugation, and the cell paste frozen at -20°C.

B) Culture media

1) Glucose peptone beef infusion (GPBI)

Infuse one pound of well minced fat-free beef in 1.0 l of tap water for 24 hours in the refrigerator. Then, infuse at 60–65°C for 45 min in a double boiler. Strain infusion through a double thickness of cheese cloth. Boil filtrate for 2–3 min. Cool Filter through Whatman #1 filter paper and make up volume to 1 l Add:

- 5g NaCl
- 10g Peptone
- 2g Na₂HPO₄·12H₂O
- 1g Na Thioglycollate

Dissolve ingredients by boiling, and adjust pH to 7.8. Dispense into 40 x 250mm tubes and add 10% air dried meat to a final volume of 30 ml. Autoclave at 15 lb for 30 min. 50% glucose is added to the medium to a final concentration of 2% sugar. [1 lb meat yields 150 tubes].

2) Semi-synthetic medium (300 ml)

- 3g peptone
- 3g beef extract
- 1.5g yeast extract
- 1.5g cysteine HCl
- 1.5g Sodium acetate
- 0.3g Starch
- 0.6g agar
Dissolve in 300 ml dH$_2$O, autoclave; boil before use, supplement with 50% glucose to a final concentration 2%, cool, inoculate with the entire 30 ml GPBl culture.

3) Synthetic medium (3 l and 30 l)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>60g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.3 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.3 g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>46.8 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>4.2 g</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>6.0 g</td>
</tr>
</tbody>
</table>

Dissolve in 3 l dH$_2$O, autoclave; add:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>biotin</td>
<td>3 µg</td>
</tr>
<tr>
<td>para-aminobenzoic acid</td>
<td>3 µg</td>
</tr>
<tr>
<td>FeSO$_4$</td>
<td>30 mg</td>
</tr>
</tbody>
</table>

Cool, and inoculate with the entire 300 ml culture.

The same procedure is followed for the production of the 30 l cultures, using the entire 3 l culture as inoculum.

C) Purification of Fd

The frozen cell paste from one 30 l culture is thawed overnight at 4°C. 600 ml of cold dH$_2$O are added and the suspension is stirred for 1 h. 600 ml of cold acetone are then added and stirring is continued for 10 min. The lysate is centrifuged for 30 min in a
Sorval GSA rotor at 9 k and 4°C. Stainless steel bottles are used as all available plastic bottles are weakened by the acetone in the extract. The supernatant is added to an ion exchange column. Two methods are described:

1) **Purification on DEAE-cellulose (followed by Sephadex G-50)**
   a) Preparation of DEAE-cellulose

   The dry cellulose is swollen in dH₂O followed by the following washes: twice in .1 in NaOH; twice in dH₂O; twice in .1 M HCl; twice in dH₂O; twice in .1 M NaOH; twice in dH₂O and all fines are removed. Pour two 25 x 50 x 200 mm columns. Equilibrate with 100 ml 1.0M phosphate buffer pH 6.5 and wash with 500 ml dH₂O (each column).

   b) Running the columns

   Add the acetone extract at room temperature and discard the yellow effluent. Wash with 250 ml dH₂O, discard the effluent. Wash with 0.15M Tris-HCl pH 7.3 until effluent's absorbance at 260 nm is less than 0.10 units. Elute with 0.65M NaCl in 0.15M Tris-HCl pH 7.3 in a minimal volume.

   c) Ammonium sulfate precipitation

   Desalt eluate by dialysis using Spectraphor dialysis tubing with an exclusion of 2,000 M.W. (This may be done under cold, running tap water) Precipitate with solid (NH₄)₂SO₄ to 60% at 4°C for 6 hr. Centrifuge in Sorvall SS34 rotor at 19K and 4°C for 30 min. Discard the pellet. Re-precipitate the supernatant by increasing the (NH₄)₂SO₄ concentration to 90% saturation; let precipitate to form overnight at 4°C. Centrifuge in a Sorval SS34 rotor at 19K and 4°C
for 30 min and discard the supernatant. Dissolve pellet in PBS, dialyze overnight into PBS. Concentrate on Polyethylene glycol molecular weight 22,000.

d) Sephadex column purification

Load onto a Sephadex G-50 column (30 x 1000 mm), 2-3 ml Fd-containing sample.

Run at room temperature in PBS + 0.02% NaN₃. Collect 2-3 ml fractions. Read absorbance at 280 nm and 390 nm. Pool fractions with highest A₃₉₀nm : A 280 nm ratio.

2) Purification using DEAE–Sephadex (Sephadex A25)

a) Preparation of Sephadex A-25 (Pharmacia, Sweden).

Swell gel in dH₂O for 3 hr. Wash 2 x in 0.01 M NaOH in a 500 ml scintilled glass funnel under mild vacuum; wash twice in dH₂O; twice in 0.1 HCl; twice in dH₂O; twice in 0.01 M NaOH; twice in dH₂O, and pour off fines. Equilibrate with 1.0 M phosphate buffer pH 6.5 by gently stirring gel in funnel and letting sit about 5 min. Wash extensively with H₂O. Always use light vacuum and do not let gel bed dry out.

b) Purification of Fd.

Apply supernatant of cell lysate to A-25 in a 500 ml scintilled glass funnel under mild vacuum. Wash with 500 ml dH₂O to remove the acetone. Wash with 0.15 M Tris–HCl buffer at pH 7.3 until most of the yellow colour disappears (2–4 l). Wash with 0.15 M Tris–Cl buffer pH 7.3 supplemented with 0.15 M NaCl until A₂₆₀nm of the effluent is less than 0.10 units. Elute with 0.15 M Tris–HCl + 1.0 M NaCl pH 7.3.
c) Concentration and desalting of Fd

Dilute the eluate 1:1 with dH$_2$O and load into a large capacity Amicon ultrafiltration device with a UM2 membrane. The resulting Fd may be run in Sephade G-50 in order to ascertain purity.

3) Characterization of Sephadex A-25

The use of A-25 for purification of Fd was assessed by the following experimental set-up: Set up 10 test tubes with 0.5g packed A-25. To each tube add 1.5 ml of the following buffers.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Buffer Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.15 M Tris-HCl pH 7.3</td>
</tr>
<tr>
<td>2</td>
<td>&quot; + 0.0075125 M Nacl</td>
</tr>
<tr>
<td>3</td>
<td>&quot; + 0.015625</td>
</tr>
<tr>
<td>4</td>
<td>&quot; + 0.03125</td>
</tr>
<tr>
<td>5</td>
<td>&quot; + 0.0625</td>
</tr>
<tr>
<td>6</td>
<td>&quot; + 0.125</td>
</tr>
<tr>
<td>7</td>
<td>&quot; + 0.25</td>
</tr>
<tr>
<td>8</td>
<td>&quot; + 0.5</td>
</tr>
<tr>
<td>9</td>
<td>&quot; + 1.0</td>
</tr>
<tr>
<td>10</td>
<td>&quot; + 2.0</td>
</tr>
</tbody>
</table>

To each tube, add 0.5 ml crude Fd (final $A_{390nm} = 1.31$ units/ml). After 5 min, the tubes are centrifuged and the absorbance read. The results are recorded in Figure 35; it can be seen that a wash with both (A) 0.15 M Tris-HCl pH 7.3 and (B) 0.15 M Tris-HCl + 0.15 M HCl, pH 7.3 eliminates material with very low $A_{390nm}$ absorbance, whereas elution (C) 1.0 M NaCl in the buffer removes almost entirely all $A_{390nm}$ material.
Figure 35. Fd elution profile of Sephadex-A25 with NaCl. 0.5 g A-25 in 0.15 M Tris-NCl pH 7.3 with increasing concentrations of NaCl were tested for Fd binding to DEAE-Sephadex (A-25).
4) Comparison of the two methods of purification.

The lysate supernatant from one cell paste was split in two and then purified by the two methods. The amount of Fd was calculated and the ratio determined. Unfortunately, at this time we were working with an extremely unstable preparation of Fd i.e. one whose ratio $A_{390nm} : A_{289nm}$ was dropping and aggregation was very apparent. This aggregation could be observed on analysis by slab-gel electrophoresis where bands were visible through the gel at log intervals. The bands were cut out, eluted by incubation in ELISA coating buffer and tested in ELISA with monoclonal antibody. All bands were found to react (data not shown). Nevertheless the two methods of Fd purification could be compared. It can be seen from table VIII that slightly more Fd was obtained by A-25 purification, and the ratio was also superior.

Further comparison of the two preparations was undertaken by Sephadex G-50 chromatography. The elution profiles for both preparations are demonstrated in figure 36 A and B below. It is very clear that A-25 purification eliminates the heavy contaminant peak almost entirely. Purification on DEAE-cellulose and ammonium sulfate cuts produces an extremely large contaminant peak at the column void volume. This peak was shown in early parts of this research to contain a polyclonal activator of lymph node cells.

5) Advantages of method B over method A

Method B, purification of lysate supernatants on A-25, can be performed from start to finish in 4 hours with a product usually superior
Table VIII - Comparison of yields by two methods of Fd purification: DEAE-cellulose and Sephadex A-25

<table>
<thead>
<tr>
<th>Method</th>
<th>Amount recovered</th>
<th>A_{390nm}/A_{280nm}</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE cellulose</td>
<td>38.09 mg</td>
<td>0.146</td>
</tr>
<tr>
<td>Sephadex A-25</td>
<td>46.20 mg</td>
<td>0.263</td>
</tr>
</tbody>
</table>
Figure 36. Comparison of two methods of Fd purification on G-50. Fd originally purified on A) DEAE-cellulose and by (NH$_4$)$_2$SO$_4$ precipitation, and B) on Sephadex A-25, was tested for purity on Sephadex G.50.
to that produced by method A: DEAE-cellulose, followed by (NH₄)SO₄ 60% and 90% cuts, followed by G-50 chromatography. Method A takes 4 to 5 days. Use of A-25 therefore results in a considerable saving of time and effort. Method B is also much more economical in terms of reagents and buffers. With method A, there is a lot of loss due to the manipulations required at every step. The yield at the end of the two methods may differ 5-10 fold. Considering that the yield in already better with A-25 as compared to DEAE-cellulose, it is easy to see how losses accumulate with subsequent steps.

D. Quantitation of Fd.

There are many techniques for the quantitation of Fd, some are rapid but just rough estimates, others are time consuming, yet quite accurate. Listed in table IX are the techniques, their advantages and disadvantages.

Thanks is given to D. Demetrick for suggesting this much improved technique of Fd purification.
<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance at 390nm</td>
<td>Rapid, estimates purity of sample, using ratio of A390:A280nm</td>
<td>Depends on Fe³⁺⁺⁺ on Fd which under certain conditions is no longer present or at another oxidation state</td>
</tr>
<tr>
<td>Amino acid analysis</td>
<td>extremely sensitive</td>
<td>tedious, time consuming dependent on good technique and apparatus</td>
</tr>
<tr>
<td>ELISA</td>
<td>rapid</td>
<td>need standard. No indication of purity</td>
</tr>
<tr>
<td>HPLC</td>
<td>extremely sensitive will pick up oxidized products</td>
<td>too sensitive, minor contaminants may mask quantitation</td>
</tr>
<tr>
<td>Biuret or Folin protein assays</td>
<td>none</td>
<td>low Fd reactivity by any of these methods.</td>
</tr>
<tr>
<td>Biorad microassay</td>
<td>rapid and economical if performed in microtitre plates which can be subsequently read on Titretek with an appropriate filter.</td>
<td>no assessment of purity</td>
</tr>
</tbody>
</table>
III Purification of "N" and "C" from digests

A. Elimination of carboxypeptidase A from digests.

CPA was eliminated from digests using an immunoabsorbent. A rabbit was immunized with 50 μg CPA in CFA at monthly intervals for three months and bled at seven, fourteen and twenty-one days after the last immunization. The titre, measured by ELISA, of the pooled sera was 1:200,000. A five ml aliquot of the serum was precipitated on ice with (NH₄)₂SO₄ at 50% final concentration. The precipitate, which contained all the antibody activity, was centrifuged, dissolved, and dialyzed against citrate-phosphate-buffer at pH 8.0. The antibody was coupled to affigel-10 [Bio-rad] by the method described in the manufacturers instructions.

On ice:
- 3.0 ml fully resuspended beads
- wash 3 x with 2-propanol (do not let gel go dry, work rapidly)
- wash 3 x with 4°C dH₂O and transfer to test tube
- add 3.0 ml antibody to gel
- rotate at 4°C for 4 hr.
- unreacted sites blocked for 1 hr. at 4°C with monoethanolamine
  pH 8.0, 10% final concentration.

The gel was loaded onto a column, washed exhaustively and tested for CPA and Fd absorption. The results are shown in figure 37A. 20 ml
Figure 37. Purification of "N" and "C" fragments from enzyme digests

A) Rabbit anti-carboxypeptidase A was linked to Affigel-10 to form an immunoadsorbent. CPA absorption profile is shown. B) Carboxymethyl Sephadex was used to purify TR out of the digest. An elution profile is shown.
A CARBOXYPEPTIDASE A IMMUNOABSORBENT

B CM-SEPHADEX
of CPA at 0.151 units/ml at $A_{280\text{nm}}$ was loaded on. The column was washed with 10.0 ml Borate-saline buffer and eluted with 0.1 M HCl.

B. Elimination of trypsin from digests.

Rabbits were immunized with 50 µg trypsin in CFA according to the protocol described for CPA above. Rabbits made no anti-trypsin antibody. Therefore an alternate route for this separation was considered. As the pi of trypsin is 10.5 and Fd is 3.7, cation exchange chromatography could be used. Carboxymethyl Sephadex (Sephadex C-50, Pharmacia) was chosen.

1. Preparation of Sephadex C-50

   Well gel in dH$_2$O

   Wash twice in 0.01 M NaOH

   twice in dH$_2$O

   twice in 0.1 M HCl

   twice in dH$_2$O

   twice in 0.01 M NaOH

   twice in dH$_2$O

   load the column

2. Testing and running the column

   No binding of Fd was observed to C-50. To test the activity of C-50, 1.0 ml of trypsin at 0.64 units/ml at $A_{280\text{nm}}$ was loaded in digestion buffer (0.046 M Tris + 0.0115 M CaCl$_2$ + 10 mM 2-mercaptoethanol buffer at pH 8.1). The column was washed with 0.2 M
acetate buffer pH 5.0 (10 ml) and eluted with 0.1 M NaOH. The results are shown in figure 37 B. After the use of either the CPA immunoabsorbent or C-50, the column effluent containing the desired fragment of Fd is collected and dialyzed in Spectaphor dialysis tubing into the appropriate buffer to eliminate any peptides and concentrated on Polyethylene-glycol MW 22,000.
IV. Detection by ELISA of anti-Fd antibodies produced by primary and secondary splenocyte cultures.

The cellular ELISA was established (153) as an alternative to the Cunningham plaquing assay (283). A study was performed in order to determine whether primary cultures of splenic cells could be assayed for anti-Fd antibody-producing ability and to determine the limits of detection of this assay in the study of high responder animals versus intermediate antibody producers. In the extensive experiment described below in tables X and XI, a number of pertinent parameters were addressed.

Both immune and non-immune B10.BR mice were sacrificed, their splenic lymphocytes were cultured in RPMI 1640 + 3.5 g/l NaHCO$_3$ + 10 mM HEPES + 5x10$^{-5}$M 2-mercaptoethanol + 10% FCS final concentration and with or without Fd at 10 μg/ml final concentration (previously shown to be optimal; 1,3,10 and 33 μg/ml had been tested) 2x10$^7$ cells per 2.5 ml Linbro well cultured for 4 or 5 days, were harvested, and their viability assessed (Table XI). The cells were loaded onto ELISA plates coated with or without Fd at 10 μg/ml. Identically set up plates were incubated with the cells for one, two or three days before the ELISA was performed. The enzymatic reaction was measured after 2.0 hr. The results from all cultures were compared and described below; each value is an average of quadruplicate tests. The net results between background binding and Fd binding are shown. The following conclusions may be reached from these and other data not shown:
Table X - Cellular ELISA with B10.BR spleen cells

<table>
<thead>
<tr>
<th>Primary a) Culture</th>
<th>ELISA</th>
<th>1 day incubation</th>
<th>2 day incubation</th>
<th>3 day incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 day priming c)</td>
<td>5 day priming c)</td>
<td>4 day priming c)</td>
</tr>
<tr>
<td>Cell Source Fd</td>
<td></td>
<td>4 day priming c)</td>
<td>5 day priming c)</td>
<td>4 day priming c)</td>
</tr>
<tr>
<td>non- 1x10^6</td>
<td>.112</td>
<td>.025</td>
<td>.132</td>
<td>.080</td>
</tr>
<tr>
<td>immune + 5x10^6</td>
<td>.069</td>
<td>.087(†)</td>
<td>.073</td>
<td>.124(†)</td>
</tr>
<tr>
<td>immune - 5x10^6</td>
<td>.123</td>
<td>.119</td>
<td>.175</td>
<td>.136</td>
</tr>
<tr>
<td>immune + 5x10^6</td>
<td>.041</td>
<td>.226(†)</td>
<td>.185</td>
<td>.110</td>
</tr>
<tr>
<td>b) Primed - 5x10^5</td>
<td>.038</td>
<td>.055</td>
<td>.068</td>
<td>.065</td>
</tr>
<tr>
<td>primed + 5x10^5</td>
<td>.042</td>
<td>.107(†)</td>
<td>.156(†)</td>
<td>.007</td>
</tr>
<tr>
<td>primed - 1x10^6</td>
<td>.089</td>
<td>.097</td>
<td>.169</td>
<td>.151</td>
</tr>
<tr>
<td>primed + 2.5x10^6</td>
<td>.072</td>
<td>.126(†)</td>
<td>.168</td>
<td>.214†</td>
</tr>
<tr>
<td>primed + 5x10^6</td>
<td>.130</td>
<td>.145</td>
<td>.235</td>
<td>.168</td>
</tr>
<tr>
<td>primed - 2.5x10^6</td>
<td>.139</td>
<td>.202†</td>
<td>.257(†)</td>
<td>.372†</td>
</tr>
<tr>
<td>primed + 5x10^6</td>
<td>.043</td>
<td>.120</td>
<td>.268</td>
<td>.327</td>
</tr>
<tr>
<td>primed +</td>
<td>.168†</td>
<td>.332†</td>
<td>.542†</td>
<td>.266</td>
</tr>
</tbody>
</table>

Total culture time 5 6 6 7 7 8

a) 2.5 ml cultures with 2x10^7 cells, plus or minus Fd at 10 μg/ml.
b) primed mice were used which were rested one month.
c) net results are shown i.e. ELISA reactivity on Fd minus reactivity in uncoated wells. Average of four wells. Values are the absorbance reading at 405 nm.
† significant result
(†) low but measurable antibody
Table XI - Cell recoveries from B10.BR cell cultures

<table>
<thead>
<tr>
<th>Duration of primary culture</th>
<th>Source of culture with Fd</th>
<th>% cell recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.I. a)</td>
<td>-</td>
<td>62.9</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>50.5</td>
</tr>
<tr>
<td>Primed</td>
<td>-</td>
<td>41.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>42.9</td>
</tr>
<tr>
<td>N.I.</td>
<td>-</td>
<td>37.5</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>39.1</td>
</tr>
<tr>
<td>Primed</td>
<td>-</td>
<td>41.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>66.8</td>
</tr>
</tbody>
</table>

a) N.I. = non-immune animals
1) Culturing unprimed cells for a primary \textit{in vitro} response is successful only in the 8 day assay (total culture time: 5 day priming culture + 3 days on ELISA). It is suggested that extending the primary culture to six or seven days may be more advantageous, however the cell recoveries may be lower as compared to cell recoveries in four and five day cultures as seen in table XI.

2) With cultures of \textit{in vitro} primed cells, adequate responses are observed within six day cultures (total culture time); with lower cell numbers, a longer time is needed; 7 to 8 days in culture and $2.5 \times 10^6$ cells per well are the optimal conditions. No clear difference exists between the relative lengths of culture times (\textit{in vitro} primary) and ELISA incubation.

3) Background sticking to the ELISA plate was found to increase with cell number, but more dramatically with the overall incubation time, the length of the ELISA incubation time being the largest contributing factor to background.

4) An Fd concentration of 10 $\mu$g/ml in the ELISA culture was optimal with $1 \times 10^6$ cells per well.

5) The use of this assay for studying intermediate responders was determined with a further experiment. (CBA X DBA/2) $F_1$ spleen cells from primed or non-immune mice were set up in culture with no antigen, Fd or PPD at 10 $\mu$g/ml and incubated for 6 days then incubated on ELISA plates for 3 days on Fd or BSA at 10 $\mu$g/ml. Results of this experiment are shown in table XII and indicate that
Table XII - Cellular ELISA with (CBA x DBA/2) F1 spleen cells

<table>
<thead>
<tr>
<th>Priming culture</th>
<th>Culture antigen</th>
<th>Fd</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>non immune</td>
<td>Fd</td>
<td>0.500</td>
<td>0.461</td>
</tr>
<tr>
<td></td>
<td>PPD</td>
<td>0.603</td>
<td>0.461</td>
</tr>
<tr>
<td>immune</td>
<td>–</td>
<td>0.708</td>
<td>0.414</td>
</tr>
<tr>
<td></td>
<td>Fd</td>
<td>1.124</td>
<td>0.440</td>
</tr>
<tr>
<td></td>
<td>PPD</td>
<td>0.720</td>
<td>0.240</td>
</tr>
</tbody>
</table>
this cellular ELISA may be used for culture of intermediate responding cells but an increase in the incubation period is required.
V. Important parameters of the proliferation assay

Beyond proper immunization and execution of the proliferation assay using a modification of the protocol described by Lee et al. (256), it was found that two parameters are critical for the maximalization of the assay: the processing and sterilization of Fd and the choice of the supplementing human serum.

A. Processing and sterilization of Fd

It was found that if Fd was purified as was originally specified by Tanaka et al. (148), a "polyclonal stimulator" contaminant was present in some preparations as seen in data presented in table XIII. This problem of non-immune cells proliferating to Fd was solved by passing the Fd over G50-Sephadex columns. The "mitogenic" material was eluted close to the void volume. The new technique of purifying Fd by Sephadex A-25 eliminates this problem as well.

A problem of greater consequence was discovered with Fd during sterilization by Millipore filtration (0.22 um). This may be due to the fact that Fd, being very electronegative, binds to the millipore membrane, effectively reducing the critical antigen concentration, or that native Fd is aggregated to such an extent that it will not filter. Table XIV shows the comparison between millipore sterilized Fd and Fd sterilized by microwave radiation. As is shown in Figure 38, Fd is stable to microwave irradiation for 30 seconds as measured by ELISA; Fd sterilized for 30 sec and used in long term cultures (greater than 2
Table XIII - Proliferation of B10.S lymph node cells

<table>
<thead>
<tr>
<th>mouse</th>
<th>&quot;Mitogenic&quot; Fd</th>
<th>Pure Fd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+FΔ</td>
<td>-FΔ</td>
</tr>
<tr>
<td>boosted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11,933</td>
<td>603</td>
</tr>
<tr>
<td>2</td>
<td>13,830</td>
<td>953</td>
</tr>
<tr>
<td>3</td>
<td>8,410</td>
<td>240</td>
</tr>
<tr>
<td>4</td>
<td>8,673</td>
<td>290</td>
</tr>
<tr>
<td>5</td>
<td>6,020</td>
<td>250</td>
</tr>
<tr>
<td>6</td>
<td>12,677</td>
<td>233</td>
</tr>
<tr>
<td>non-immune</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8,397</td>
<td>260</td>
</tr>
<tr>
<td>2</td>
<td>10,293</td>
<td>273</td>
</tr>
</tbody>
</table>
Table XIV - Effect of antigen sterilization technique on the proliferation assay

<table>
<thead>
<tr>
<th>Fd conc. (µg)</th>
<th>Millipored Fd</th>
<th>Microwaved Fd b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>immune(S.I.)</td>
<td>non-immune(S.I.)</td>
</tr>
<tr>
<td>12.5</td>
<td>0.68</td>
<td>1.48</td>
</tr>
<tr>
<td>25.0</td>
<td>2.91</td>
<td>2.22</td>
</tr>
<tr>
<td>50.0</td>
<td>8.48</td>
<td>2.49</td>
</tr>
<tr>
<td>100.0</td>
<td>10.02</td>
<td>1.50</td>
</tr>
</tbody>
</table>

a) primed and boosted (7 day) B10.BR lymph node cells were used.

b) 30 second microwave pulse.
Figure 38. Microwave sterilization of Fd for cultures. Fd in media (250 μg/ml, 2 ml) was tested for sensitivity to microwave irradiation by testing treated Fd on ELISA with 6-90 monoclonal anti-Fd antibody. Fd microwaved for 30 sec was found sterile for purposes of long term tissue culture.
weeks) was not responsible for any contamination (yeast or bacterial). The above observation may partially explain why the proliferation assay stopped working by mid-1979. In the early experiments, NEM-Fd was chosen as antigen due to a previous observation that it was the most effective T-cell stimulator (12). This modification of Fd (described in detail by Gregerson et al (12)) seems to de-aggregate Fd, permitting filtration. From the time native Fd was used, low or no stimulation at all was observed.

B. Selecting culture conditions

It was suggested that maintenance of culture pH is critical to the success of the assay. Therefore, as can be seen in Table XV, the addition of HEPES was tested. Serum concentration and source were also compared. It may be said that HEPES buffering provides no significant improvement to the assay, while choice of human serum is critical (in this experiment there was no real difference between a 5% and 10% supplement).

C. Selection of human serum

As can be seen in Table XVI, the source of serum is critical in obtaining low background proliferation and good specific stimulation. Some sera have a naturally high background (e.g. L.S.), which may be attributed to a high anti-mouse antibody component, as cellular agglutination is observed. Other undetermined factors may play a role in the unsuitability of some sera. As a general rule, outdated plasma may be obtained from Red Cross; the serum is drawn-off after coagulation, aliquoted, heat inactivated and stored at -70°C. A number of such sera
Table XV - Culture conditions

<table>
<thead>
<tr>
<th>Hepes conc.</th>
<th>Serum&lt;sup&gt;a)&lt;/sup&gt; source</th>
<th>% serum</th>
<th>Culture with PPD Cpm+PPD</th>
<th>Cpm-PPD</th>
<th>S.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A</td>
<td>10</td>
<td>58,297</td>
<td>50</td>
<td>1166</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>52,537</td>
<td>93</td>
<td>563</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>10</td>
<td>680</td>
<td>43</td>
<td>16</td>
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<tr>
<td>0.005M</td>
<td>A</td>
<td>10</td>
<td>57,327</td>
<td>77</td>
<td>747</td>
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<tr>
<td></td>
<td></td>
<td>5</td>
<td>81,580</td>
<td>250</td>
<td>326</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>10</td>
<td>50</td>
<td>53</td>
<td>1</td>
</tr>
<tr>
<td>0.01M</td>
<td>A</td>
<td>10</td>
<td>90,673</td>
<td>107</td>
<td>850</td>
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<tr>
<td></td>
<td></td>
<td>5</td>
<td>86,577</td>
<td>397</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>10</td>
<td>53</td>
<td>63</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a)</sup> A is a Red Cross outdated serum.

<sup>b)</sup> is a pool obtained from volunteers in the laboratory.
<table>
<thead>
<tr>
<th>Source of human serum</th>
<th>Background cpm</th>
<th>Fd specific cpm</th>
<th>PPD specific cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.S.</td>
<td>34,225</td>
<td>31,658</td>
<td>35,288</td>
</tr>
<tr>
<td>lab pool</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>284</td>
<td>11,754</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>672</td>
<td>N/A</td>
<td>1,177</td>
</tr>
<tr>
<td>3</td>
<td>253</td>
<td>56,033</td>
<td>27,367</td>
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<tr>
<td>Red Cross</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>123</td>
<td>11,890</td>
<td>58,297</td>
</tr>
<tr>
<td>B</td>
<td>25,233</td>
<td>88,220</td>
<td>128,220</td>
</tr>
<tr>
<td>C</td>
<td>393</td>
<td>40,815</td>
<td>92,095</td>
</tr>
<tr>
<td>D</td>
<td>977</td>
<td>5,730</td>
<td>131,183</td>
</tr>
<tr>
<td>J.D.</td>
<td>700</td>
<td>2,230</td>
<td>128,240</td>
</tr>
<tr>
<td>E.B.</td>
<td>807</td>
<td>33,857</td>
<td>27,280</td>
</tr>
</tbody>
</table>
ought to be pretested in the proliferation assay, and a selected batch used for all related assays. In the interest of "stretching" a good batch of serum, all manipulations with the cells should be done in PBS + 5% FCS, the cells resuspended in unsupplemented RPMI-1640, counted, dispensed into microtitre culture plates, and only at that time that the serum is added.
VI Cyclophosphamide treatment of non-responder mice

A preliminary study was done in an attempt to elucidate the non-responsiveness status of H-2\textsuperscript{d} strains. Cyclophosphamide is a selective immuno-suppressive agent which is toxic to cells as it alkylates DNA. Its effects on cells of the immune system vary with the dose; at a low dose, there is evidence that T-suppressor cells are preferentially inactivated, while at higher doses both helper and suppressor compartments are inactivated. It was thought that if T-suppressor cells were responsible for H-2\textsuperscript{d} non-responsiveness to Fd, cyclophosphamide treatment might alter this status. Mice (DBA/2) were given a dose of cyclophosphamide of 50 mg/kg, 3 days before the primary immunization. The treatment had no effect on serum antibody levels. However, when cells from mice treated with cyclophosphamide before each immunization with Fd were put into 9 day (total) ELISA culture with Fd (see appendix IV), a small amount of antibody was generated by spleen cells from treated mice. These preliminary results indicated the need for more extensive studies where dose effects could be analyzed, for one. As no serum antibody was observed in these mice, one could tentatively submit that cyclophosphamide had no effect in removing the putative suppressor cell in H-2\textsuperscript{d} mice.
VII. The use of Fd-lysozyme conjugates

Another line of reasoning was employed in the relief of the non-responsiveness status. It was predicted that if H-2\textsuperscript{d} mice either lack the appropriate T-helper cells or that Fd induces suppression in these mice, conjugation of Fd to an immunogenic carrier should counteract this "lesion" in non-responder mice. Lysozyme was chosen as it is immunogenic in H-2\textsuperscript{d} mice, it is a well-defined antigen, and it is electrostatically opposite to Fd (HEL, pI 10.5; Fd, pI 3.7). Two methods were devised to obtain Fd-HEL conjugates with linkage through cysteine residues (in order to leave N- and C- termini of Fd free).

Method A

A. Reduction of HEL (M.W. 13,000)

10 mg HEL dissolved in 0.55 M Tris-HCl pH 8.1 + 15 times molar excess of dithiothreitol

Wash precipitate

Resuspend in 0.5 ml Dimethyl sulfoxide.

B. Preparation of N,N'-p-phenylenedimaleimide (M.W. 268.2) at 10 times molar excess of cysteines (8 Cys in HEL)

For 10 ml HEL need 0.0825 g DPDM dissolved in 1 ml DMSO

Add HEL dropwise over 1 hour to 1.0 ml DPDM 30 \mu l at a time, stirring

Continue stirring for 36 hrs.
C. Reduction of Fd

To 5.0 ml Fd (0.49 mg/ml) add 0.264 ml 100% TCA
Add 100 μl 2-mercaptoethanol
Centrifuge, wash twice in 95% ethanol
Dissolve in 1.0 ml DMSO

D. Product formation

Add 1 ml 0.05 M Tris-HCl pH 8.0 to precipitate HEL/DPDM, wash and resuspend in 1.0 ml DMSO
Add HEL to Fd in 0.1 ml aliquots, stirring
Continue stirring for 48 hours.
Add 5.0 ml 0.05 M Tris-HCl pH 8.0 to precipitate product
Wash pellet 2 times
Resuspend in 1.0 ml 0.05M Tris-HCl pH 8.0

Method B

A. 20 mg of HEL was dissolved in 2.0 ml 10.0 M urea
Add 40 μl of 2-mercaptoethanol
Adjust pH to alkaline with NH₄OH
Flush with N₂
Incubate at 37°C for 4 hrs

B. S-aminoethylation of reduced HEL
Add 40 μl of ethylene imine at 10 minute intervals
Pass over 1.5 cm x 27.5 cm G-10 column, in 0.2N acetic acid
Pool peak fractions, concentrate on AMICON PM10

Concentrate into 1.0M Borate buffer pH 8.2

C. TCA-Fd

15 mg Fd in 5.0 ml dH₂O precipitated with 0.264 ml 100% TCA + 0.200 ml 2-mercaptoethanol and EDTA.

Under N₂, stirr for 1 hr at 4°C

Centrifuge

Wash precipitate in twice 95% ethanol

D. Blocking the NH₂-terminal and lysine of TCA-Fd

Dissolve in TCA-Fd precipitate (10 mg) 1.0 ml 1.0 M Borate buffer pH 8.2 at room temperature, stirring add 0.100 ml 2-mercaptoethanol

Add 10 μl aliquots of citraconic anhydride every 30 min to 80 μl

*Maintain at pH 8.2 with NaOH

Stir for 2 more hours maintaining the pH. Dialyze in spectraphor 6 dialysis tubing at 4°C against dH₂O at pH 8.5-8.8 (with NH₄OH) for 4 hr; change water.

*Never let pH drop below pH 8.0

Proceed with next part immediatly

Concentrate on Amicon UM2 into 1.0M Tris buffer pH 8.6

E. S-aminoethylation of blocked TCA-Fd

For 10 mg Fd in 1.0 M Tris buffer pH 8.6

3 additions of 50 μl aliquots of ethylene-imine at 10 minute intervals.
Run over G-10 column equilibrated with 1.0M Tris buffer pH 8.6
Concentrate into 1.0 M borate buffer pH 8.2

F. Reaction with gluteraldehyde
10 ml of 15% gluteraldehyde was placed in vial with stirring rapidly
(add 1 drop 3 M NaOH)
Aminoethylated Fd in borate buffer was added slowly dropwise
Stir for 2 hrs at 25°C
Dialyze against 2 changes of dH₂O pH 8.6
Concentrate
Dialyze against 0.1 M Tris Buffer pH 8.6

G. Add S-aminoethylated HEL in 1.0 ml aliquots to the blocked,
S-aminoethylated, gluteraldehyde treated reduced Fd.
Stir rapidly-get flocculant conjugate
Centrifuge and resuspend in borate buffer and 10% monoethanolamine pH
8.2 to 1% final concentration.

Results of immunization of B10.D2 (non-responder) and B10.BR (high
responder) mice are shown in table XVII. Neither method resulted in a
product that is immunogenic in B10.D2; both products are immunogenic in
B10.BR, indicating that non-responsiveness cannot be alleviated in the
classical hapten-carrier system. However, the problem may stem from the
fact that these manipulations involve the breakage of disulfides in HEL,
bonds that are extremely important in conformational maintenance of
residues in proximity of one another in the antigenic determinants. As
can be seen in experiment A, both strains make a very low anti-HEL
Table XVII - Use of Fd-lysozyme conjugates

<table>
<thead>
<tr>
<th>Experiment using conjugate</th>
<th>Strain</th>
<th>Immunizing antigen</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fd</td>
<td>HELc)</td>
</tr>
<tr>
<td>A</td>
<td>B10.BR</td>
<td>Fd + HEL</td>
<td>0.237 ± .023</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fd - HEL</td>
<td>0.411 ± .029</td>
</tr>
<tr>
<td></td>
<td>B10.D2</td>
<td>Fd + HEL</td>
<td>0.020 ± .011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fd - HEL</td>
<td>0.011 ± .003</td>
</tr>
<tr>
<td>B</td>
<td>B10.BR</td>
<td>Fd - HEL</td>
<td>0.783 ± .365</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CFA</td>
<td>0.001 ± .001</td>
</tr>
<tr>
<td></td>
<td>B10.D2</td>
<td>Fd - HEL</td>
<td>0.024 ± .006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CFA</td>
<td>0.001</td>
</tr>
</tbody>
</table>

a) Method A. Mice were immunized, and boosted, sera were obtained 9 days after the secondary and tested at 1/100 on Fd and HEL at 4 µg/ml and Fd-HEL 1:400 (equivalent approx. to 4 µg/ml Fd).

b) Method B. Mice were finally boosted with 200 µg of the conjugate as two previous immunizations yielded no response. The mice were bled 7 days after the tertiary.

c) reduced HEL is used as the control.
response; this could mean that very low levels of immunogenic HEL are present in the conjugate. This situation most probably does not eliminate potential T-helper determinants, indicating that non-responsiveness in H-2^d mice may not be counteracted by this particular "hapten-carrier" conjugate. The question that remains to be answered is whether this phenomenon holds for other hapten-carrier systems such as Fd-KLH.

Thanks is given to D. Demetrick for his help in devising the above procedures of conjugation.
VI Preliminary studies with T-cell hybridomas

A proposal is put forward that helper and suppressor factors produced by T-cells in the response to Fd could be analyzed using T-cell hybridoma technology. Since functional assays for help or suppression of the anti-Fd response are not very sensitive (i.e. the cellular ELISA) and very time consuming, it is suggested that serologically defined factors be selected first, followed subsequently by functional characterization. Therefore, idiotype positive T-hybridoma supernatants can be screened with a rabbit antiserum to B10.BR anti-Fd antibody. A pilot experiment was carried out to assess the feasibility of such an approach.

A. Fusion partner

BW 5147.G.1.4, a thymoma of AKR origin was obtained from the Salk Institute and maintained in culture: DME + 3.4 g/l NaHCO₃ + 0.29 g/l glutamate + 0.11g pyruvate + 10 mM HEPES + Penicillin/Streptomycin + 10% selected foetal calf serum. The cells were positively selected with 2.5 μg/ml 8-Thioguanine, followed by selection on 2x, 3x, 4x of the selecting agent. Cells were maintained in exponential culture.

B. Fusion

B10.BR splenocytes from immune animals were stimulated in vitro with 6 μg/ml ConA for 3 days and fused to BW.5147 following standard hybridoma technology (284); they were plated at 2.5 x 10⁶ cells/ml with an equal amount of thymocytes as feeders. Fusion product selection was carried out with hypoxanthine, aminopterin, thymidine selective media. The cultures were given 0.1 ml fresh medium, 7 days later. At 2 weeks,
80 μl of the supernatant was added to 20 μl 10 x Carbonate buffer to ELISA plates. ELISA's were performed with rabbit anti-idiotypic (purified on idiotypic columns after they were absorbed on normal mouse Ig immunoadsorbent) and developed with goat anti-rabbit Ig-alkaline phosphatase. Five positive wells were selected on one day, and eight more were selected a few days later. These positive wells were transferred into 1.0 ml cultures. The original 5 cultures came up positive in the retesting. However when the culture volume was increased to 5.0 ml, and activity was tested, all activity was lost.