EVALUATION OF RESPONSIVENESS/UNRESPONSIVENESS PATTERNS TO GLPHE IN INBRED AND CONGENIC MOUSE STRAINS

BY

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ABSTRACT

Inbred and inbred congenic mice were utilized to study responsiveness/unresponsiveness to GLPhe. A sensitive method of detection of responsiveness to GLPhe was developed via a modified ELISA technique which detects anti - GLPhe antibodies. All inbred and inbred congenic mice used have been documented previously as responders or nonresponders to GLPhe by other assay methods and correspond to the data obtained by the ELISA assay developed. Unresponsiveness to GLPhe,as demonstrated by the inhibition of GLPhe - CGG responses by GLPhe pre - immunization as well as via administration of cyclophosphamide, strongly suggests the involvement of T_S cell induction. The induction of the T_S cell is a result of a defective antisuppressor pathway as shown by RICA analysis and the functional evaluation of the complexes and the T cells acting as their targets (6 hour serum transfer experiments). Although unresponsiveness to GLPhe is due to defects in the antisuppressor pathway, different nonresponder haplotypes may have defects at any one or more stages along this pathway. It may be suggested that it is not neccessary to implicate Is genes for unresponsiveness but that both the responsiveness and unresponsiveness states depend on the function of the antisuppressor pathway as the "master switch" of immune responses.

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Ag	= antigen
APC	= antigen presenting cell
B10	= C57Bl/10SnJ
B10.A	= B10.A/SgSnJ
(3R)	= B10.A/SgSnJ(3R)
(5R)	= B10.A/SgSnJ(5R)
(9R)	= B10.A/SgSnJ(9R)
(18R)	= B10.A/SgSnJ(18R)
BSA	= bovine serum albumin
CGG	= chicken gamma globulin
DNP	= dinitrophenyl
DTH	= delayed - type hypersensitivity
ELISA	= enzyme - linked immunosorbent assay
FCA	= Freund's complete adjuvant
Fe	= ferritin
GA	= L-glutamic acid^{60}, L-alanine^{40}
GAT	= L-glutamic acid^{60} - alanine^{30} - tyrosine^{50}
GL	= L - glutamic acid^{60}, L - lysine^{40}
GLPhe	= L - glutamic acid^{56}, L - lysine^{35}, L - phenylalanine^{9}
GPA	= guinea pig albumin
GT	= L - glutamic acid^{50} - L - tyrosine^{5}
GTL	= L - glutamic acid^{50} - tyrosine^{50} - L - lysine^{35}
6HS	= six hour sera

Ia	= I region associated
IACF	= immunoglobulin and antigen complexing factor
IFN	= gamma interferon
Ig	= immunoglobulin
i.p.	= intraperitoneal
Ir	= immune response
Is	= immune suppression
i.v.	= intraveneous
MBSA	= methylated bovine serum albumin
MHC	= major histocompatibility complex
MLR	= mixed lymphocyte reaction
NMS	= normal mouse sera
O.D.	= optical density
OVA	= ovalbumin
P_1	= parent 1
P_2	= parent 2
PBS	= phosphate - buffered saline
PETLES	= peritoneal exudate T = lymphocyte enriched cells
(Phe,G) - A --L	= (L-phenylalanine, L-glutamic acid) - DL - alanine, L-lysine
PLL	= poly - L - lysine
RFC	= rosette - forming cells
RICA	= reverse immune cytoadherence technique
SRBC	= sheep red blood cells
TCA	= trichloroacetic acid

(T,G) - A --L == (L-tyrosine, L-glutamic acid) - DL - alanine, L - lysine

T_H = T helper cells

T_HF = T helper factor

T_S = T suppressor cells

T_SF = T suppressor factor

INTRODUCTION AND LITERATURE REVIEW

(I) RESPONSIVENESS

1. Ir Genes

The immune system is a highly complex unit governed by many structural and regulatory genes. In order to respond to a wide variety of encountered antigens, the immune system must be able to identify and subsequently dispose the foreign substance. The genes which allow an organism to respond to certain antigens and subsequently regulate the immune response are termed the immune response genes (Ir genes).

The study of Ir genes began when it was observed that in any random bred population, some animals will respond to a specific antigen by mounting an antibody response against that particular antigen, while other animals will not respond.

The first work to study this phenomena was performed with random bred guinea pigs utilizing the synthetic polymer DNP - PLL. Upon immunization of the guinea pigs with DNP - PLL, two categories of animals were distinguished:

(1) one that developed DTH and a high concentration of anti-DNP antibodies and (2) a second that did not

develop DTH and developed low or no anti-DNP antibodies.

These animals that produced anti-DNP antibodies to DNP - PLL could also develop antibodies to other haptens conjugated to PLL. Since carrier recognition is necessary for the antibody response, the responder animals show their responsiveness through their T cells (Levine et al, 1963b). In the case of the nonresponder animals, their defect was shown to be in their T cell compartment, not in their B cells i.e. not a humoral defect. This was demonstrated by coupling DNP - PLL to another carrier molecule such as BSA or OVA (Green et al, 1966), which allowed the nonresponder animals to respond and produce anti-DNP antibodies. In this case however, no DTH to PLL develops.

Further experiments were performed using strain 2 and strain 13 inbred guinea pigs in which response to certain antigens occur in either strain but not concurrently in both. Since responsiveness is a dominant trait, by performing the appropriate backcrosses, the gene control could be studied to a wide variety of copolymers. It was found that strain 2 guinea pigs responded to DNP - PLL, GL, GA, low doses of BSA, and to DNP - BSA but not to GT or DNP - GPA. In the backcross of (2 X 13) F_1 with strain 13 guinea pigs, it was found that the above antigen responses were inherited together (Benacerraf et al, 1971 & Bluestein et al, 1971a). In strain 13 guinea pigs, GT and DNP - GPA responsiveness are linked (Bluestein et al,1971b). It was found that in random bred guinea pigs, responsiveness to the above antigens were not linked, although it could occur randomly in some animals.

Studies then moved into the mice where more inbred congenic

strains were available. All the inbred congenic strains used had defined genetic differences confined to the I region of their MHC so the mapping of the mouse H-2 complex as well as Ir gene mapping was possible. The types of antigens used to study Ir gene control in the mouse were synthetic antigens just as DNP - PLL was used in guinea pigs. These synthetic antigens were convenient to use because they presented only two or three distinct antigenic determinants and therefore it was possible to identify the Ir gene that regulated their response.

The branched copolymers (T,G)-A--L, (Phe,G)-A--L, and (H,G)-A--L were the first antigens used in inbred mouse strains. It was found that responsiveness to these copolymers was under a single gene control which was dominant and yielded the same results as with the single chain copolymers in the guinea pigs. Responder strains produced high IgG antibody titers directed against the branched copolymers whereas nonresponder strains produced low IgG antibodies but some IgM antibodies (McDevitt and Sela, 1965).

To this date no response to T independent antigens have been found to be governed by Ir genes (Benacerraf and Germain, 1978). Therefore, the IgG antibody of responders is a T dependent response and under Ir gene control. On the other hand, the IgM response is both a T independent and a T dependent response. In 1972, McDevitt et al mapped the Ir gene which controlled responsiveness to antigens such as (T,G)-A--L to the I region of the H-2 complex. They found that mice that responded to (T,G)-A--L could produce anti-DNP antibodies if immunized with DNP - (T,G)-A--L just as responder guinea pigs could

produce anti - DNP antibodies when immunized with DNP-PLL. Non-responder animals did not produce anti - (T,G)-A--L antibodies when immunized with this branched copolymer but these mice could be "converted" into responders i.e. could produce anti-(T,G)-A--L antibodies if immunized with (T,G)-A--L coupled to methylated BSA. Methylated BSA as mentioned before is an immunogenic carrier which is a T cell dependent antigen and since DTH reactions and helper activity that is carrier specific are both T cell functions, it was concluded that the Ir genes that are H-2 linked control responsiveness to T cell dependent antigens.

2. I Region and Subregions of Mouse H - 2 Complex

The mouse H-2 complex is located on chromosome 17 and is divided into four major regions; K, I, S, and D. The I region was first thought to be subdivided into I-A, I-B, I-J, I-E, and I-C subregions via results obtained from serological analysis of recombinant chromosome 17 (Murphy, 1981). In 1982, Steinmetz et al performed molecular cloning on the murine I region and could only identify the I-A and I-E subregions. At the DNA level, the I-C subregion was never identified and the "so - called" I-B subregion was found to be due to the effect of phenotypic loci mapping to I-A and I-E subregions (Baxevanis et al, 1982).

Immune suppression genes (Is genes) are also located within the I region. These Is genes map to the I-A and I-E subregions although the subpopulation of T cells known as suppressor T cells (T_S)

carry the alloantigen controlled by the I-J subregion. The I-J subregion's existence within the H-2 complex of the mouse is highly controversial to this date and it has yet to be located definitively in the H-2 complex. However, the I-J marker is found on T_S cells as well as on T suppressor factors.

In order to detect H-2 controlled molecules, strains which differ in the particular region or subregion of the H-2 are cross-immunized thereby producing the antibodies against the molecules in question. Using this method along with the availability of molecular probes, the I region has been found to code for a minimum of four polypeptide chains. The I-A subregion was shown to contain three genes; these being A_β, A_α, and E_β. In later studies the $A_{\beta 2}$ gene was identified 20 kb. centromeric to $A_{\beta 1}$ (Larhammer et al, 1984) and the $A_{\beta 3}$ gene was identified 70 kb. telomeric from the K locus (Devlin et al, 1984). The I-E subregion contained one gene product termed E Between the E_α and the E_β gene was another gene termed $E_{\beta 2}$ and it is still not known whether this gene is expressed. Devlin et al in 1984 identified the $E_{\beta 3}$ gene via restriction fragment polymorphisms of recombinant mouse strains and this $E_{\beta 3}$ gene was mapped to the I-E subregion. Therefore, separate genes for the six beta chains have been identified whereas only two alpha chains have been identified to date.

The alpha chains in both the I-A and I-E subregions have a molecular weight of approximately 34,000 while the beta chains have an approximate molecular weight of 28,000 daltons (Uhr et al, 1979). These two chains are associated noncovalently on B cells, antigen presenting cells, and on activated T cells and consist

of two extracellular domains, a hydrophobic transmembrane region, and a cytoplasmic domain. The beta distal extracellular segment consists of 90 amino acids held in a disulfide loop by the presence of two cysteines whereas the alpha membrane distal segment is shorter and has no disulfide loop. Both the alpha and beta membrane proximal domains form disulfide loops and their amino acid sequences are highly conserved. These domains can be considered homologous to immunoglobulin constant regions as shown by the homologies in the amino acid sequences of the alpha and beta membrane proximal domains and the Ig constant regions.

In some inbred strains, a mutation affecting the E - encoding locus results in the absence of the E_{α} chain in the cytoplasm. These strains do not express the E molecule on the cell surface since without alpha chains, the beta chains cannot integrate into the membrane (Jones et al, 1978). Here is where defects in the immune response can occur with regards to antigens which utilize gene complementation in order to get a response.

3. Ir Gene Expression

Ir gene expression is found on some B cells, and on macrophages however, the specific immune responses directed by the Ir genes are still restricted to only T dependent antigens. When an antibody response is allowed to occur by the Ir genes, helper T cells that are specific for the particular antigen in use may be stimulated. This, however, depends on the specific helper cell development and on the

ability of the local B cells to respond to the specific regulatory signals delivered by the T cells. This Ir gene control depends on both T_H cells and B cells therefore Ir gene regulation of antibody responses will only occur with T cell dependent antigens. This was demonstrated, as mentioned before, by coupling the antigen to an immunogenic T dependent carrier (Green et al, 1966). The above data demonstrate that Ir genes control specific immune responses between T and B cells. Further experimentation has demonstrated that in order for B cells to produce antibodies to the specific antigen, the helper T cells must be syngeneic or semi-syngeneic to the B cells (Katz et al, 1973a & b). The experiments performed to determine this involved priming the helper T cells with a carrier in a separate mouse of a different haplotype than the hapten primed B cells. The helper T cells which were carrier primed were then adoptively transferred into F_1 recipients. These F_1 recipient mice had been given sublethal irradiation and then the hapten - primed B cells, i.e. spleen cells from hapten-primed animals treated with anti-theta and complement, as well as the carrier primed T lymphocytes were adoptively transferred. The conjugate of hapten-carrier was injected as a challenge into these F_1 mice. The results demonstrated that for secondary antibody responses, the carrier primed T_H cells and hapten-primed B cells must be syngeneic. With respect to Ir gene controlled antibody responses to specific antigens, the F_1 T_H cells {F_1=(responder X nonresponder)} were only effective with responder antigen primed B cells. This genetic restriction of Ir gene controlled responses was shown to be due to the I-A subregion for normal antigens.

The genetic restriction of antibody responses to certain antigens required two Ir genes from two subregions. These subregions were mapped as I-A and I-E and an example of this type of antigen is a synthetic terpolymer such as GLPhe (Dorf et al, 1975). Therefore, a matching I region haplotype between T_H and B cells is necessary to elicit an antibody response to antigens that are under Ir gene control.

The macrophage also has Ia antigens present on its surface and these Ia antigens are important in the selection of T cell clone specificity as well as in antigen presentation to T cells. The presentation of antigen by macrophage to T cells has also been shown to be Ir gene restricted (Shevach et al 1972, Shevach 1976). This work was done with strain 2 and strain 13 guinea pigs receiving the antigen DNP - GL and GT as described in previous sections.

The assay used was a proliferative response measurement. They found that DNP - GL and GT induced a proliferative response in the (2 X 13)F_1 peritoneal T cells as well as when the two antigens were combined and presented by (2 X 13)F_1 macrophages. The (2 X 13)F_1 lymphocytes responded to GT if it was presented by strain 13 macrophages and to DNP - GL if it was presented by strain 2 macrophages. In the reverse combination it did not work.

Use of anti-Ia alloantisera also specifically inhibited the responses as above. This demonstrates that the macrophage Ia surface antigen is necessary for antigen presentation to the T cell as well as in the eventual antibody response which is Ir gene restricted. This macrophage Ia - antigen presentation phenomena was also shown to occur

in DTH reactions. DTH was also found to be under Ir gene control (Benaceraf et al,1967) and was subsequently mapped to the I-A subregion.

Erb and Feldman (1975) and Pierce et al (1976), using both guinea pigs and mice, found that primed T_H cells could provide help to syngeneic unprimed B cells only when the antigen presentation was performed by macrophages which were syngeneic with the T cells in the I-A subregion. This indicates that identity of the I-A subregions are important in Ir gene controlled responses and the Ia antigens play a significant role in cell interactions.

Since Ia antigens are present on macrophages and B cells, defects can occur in these cell types and these defects are termed Ir gene defects. Most Ir gene defects were detected using simple synthetic polypeptides and inbred congenic mice or guinea pigs. They were also detected at the same time that mapping of the I region of the MHC was undertaken. It was found that most of the immune responses to simple synthetic polypeptides such as GLPhe (glutamic acid^{56} - lysine^{35} - phenylalanine^{9}) were under the control of not one immune response gene but two Ir genes. This phenomena was referred to as gene complementation.

4. Ir Gene Complementation

The first antigen found to be under two gene control was the alloantigen H - 2.2 (Stimpfling and Durham,1972). By using congenic inbred strains, it was found that the ability to produce anti-H - 2.2

antibodies was dependent upon a genetic factor that is associated with the mouse's H-2K locus of the H-2^b chromosome. This genetic factor, which could be Ir-1, regulates the recipient's ability to respond to the H-2.2 which is an antigen specificity under H-2D control. There have been other systems that have demonstrated that by breeding two nonresponders together, the resulting F_1 hybrid is a responder. This shows that at least two genes are required that complement each other in the F_1 animals. An example of the above was shown with inbred congenic rats and their response to the synthetic polypeptide (H,G)-A--L (Gunther and Rude,1975). Approximately 50% of the offspring from low responders to (H,G)-A--L that were tested became high responders when crossed with intermediate responders as shown by antigen binding, quantitive precipitation tests, and antigen induced lymphocyte proliferation.

The confirmation that more than one gene is involved in the response to a specific antigen came from the study of synthetic polypeptides in inbred congenic mouse strains. The synthetic polypeptide used to achieve the clear demonstration of gene complementation has been GLPhe. In 1972, the responder haplotype to GLPhe was determined. Mice that carried the H-2 haplotype d and q were responders and mice that carried the alleles a, b, k, p, and s in their H-2 were nonresponders to GLPhe (Merryman et al, 1972). It was found that the response to GLPhe was under the control of a distinct autosomal dominant gene, similar to GAT responses, although major differences did exist in the immune responsiveness to these two synthetic copolymers.

One of the differences between GAT and GLPhe responses is in the number of strains with different H-2 haplotypes that can respond to the particular copolymer. For example,GLPhe has two H-2 haplotypes, d or q, that give a good response, whereas in the GAT system, only mice carrying the H-2 haplotype d allele were good responders. Another difference between the GAT and GLPhe system of responsiveness was the immunizing dose required to make the distinction between responders and nonresponders. In the GLPhe system, 10 ug was necessary to distinguish between responders and nonresponders, whereas with GAT, immunization of 1 ug led to a clear distinction between responders and nonresponders. GAT responsiveness was also found to be under unigenic control with the gene located in the I-A subregion whereas GLPhe responsiveness was found to be under multigenic control.

5. Coupled Ir - Gene Complementation in the GLPhe System

In 1975, Dorf and Benacerraf demonstrated that some recombinant inbred strains, i.e. mice derived from nonresponders but had an independent cross-over in their H-2 complex were responders. This suggested that a complementation of two Ir genes occured resulting in an all or none response to GLPhe. The two nonresponder parental strains originally used in the GLPhe system were A/J with either B10 or (18R). The A/J has a H-2^a haplotype i.e.I-A^kI-E^d while the B10 and (18R) have the H-2^b haplotype i.e.I-A^bI-E^b. It was shown that the mating of two nonresponders, i.e. A/J X B10 or A/J X (18R), to produce an F_1 hybrid which is a responder, requires the contribution of at

least one allele from each nonresponder. In otherwords, each of the nonresponders carry at least one gene required to elicit an immune response and both can complement the other to produce a response in the F_1 generation.

More evidence that at least two distinct Ir loci are required for responsiveness to GLPhe comes from recombinant strains. Recombinant strains such as the (3R) and (5R), both with haplotypes I-A^bI-E^k, are derived from independent recombinations or cross-over events between two nonresponder loci. In the case of both (3R) and (5R), these loci are of the H-2^a and H-2^b haplotypes. Since the (18R) and R106 strains, both of haplotype b/a i.e. I-A^bI-E^b, are non-responders to GLPhe, one of the Ir genes for response to GLPhe is localized between the I-A subregion and the S subregion i.e. in the I-E subregion. This gene was termed alpha and the chain, in strains with haplotypes b, s, and f, is only found in the cytoplasm and is not expressed on the cell surface . These strains are said not to express the alpha or I-E gene on the cell surface as well as not producing the complete E molecule therefore they are considered nonresponders to GLPhe.

By taking into account the responsiveness and cross-over points found in the (3R) and (5R) and in the (9R) and B10.HTT strains which are of haplotype I-A^sI-E^k, the positioning of the other Ir-GLPhe gene was found to be a product of the I-E subregion. This gene lies within the area defined as the I-A subregion and is termed E_β. Both of the genes coding for the alpha and beta chains are dominant therefore both the alpha and beta alleles are required for responsiveness to GLPhe.

The term gene complementation came to exist on the basis of the requirements of the alpha Ir gene and the beta Ir gene for a response. Nonresponders will carry either only one of the "responder" alleles or they will lack both of the "responder" alleles (Dorf et al, 1975 and Dorf and Benaceraf, 1975). Examples of nonresponders to GLPhe that possess only one of the responder alleles are A/J, C_3H/Hej, and B10.A. These strains possess only the alpha allele. The strains C57Bl/6, (18R), and R106 possess only the beta allele. The strains that express no responder alleles are of haplotypes s and f and examples of these strains are $C_3H.Sw$ and SJL. These are also phenotypical nonresponders to GLPhe.

By producing F_1 hybrids between GLPhe nonresponders that carry only one of the responder alleles, gene complementation is observed. In the original work produced by Dorf and Benacerraf in 1975, the mating of C57Bl/6 X SJL mice produced a F_1 containing high GLPhe responsiveness. Both the C57Bl and the SJL strain carry a beta gene but no functional alpha gene. These F_1 hybrids can be classified as GLPhe responders however they are of low responder status. In contrast to this, crossing strains which bear the haplotype $H\text{-}2^a$ with strains of the $H\text{-}2^k$ haplotype do not complement each other at all. Therefore, differences exist between alpha and beta genes of different haplotypes, for example their structure , which ultimately results in a difference in complementation ability.

The explanation given for the ability of the (B6 X SJL)F_1 hybrid to complement each other is that beta - beta complementation is also possible in addition to alpha - beta complementation (Dorf and

Benacerraf, 1975). The beta - beta complementation can also be called IA - IA complementation since it involves the I-A beta gene with the I-E beta gene, which is structurally located beside or in the I-A subregion of the mouse H-2 complex.

By using F_1 hybrids as well as recombinant mice, gene complementation with GLPhe can be studied with respect to the distinct chromosomal relationships of their alpha and beta genes as well as with respect to the cellular location of each of these complementing genes. In 1976, a number of different groups attempted to unify GLPhe complementation with the (T,G)-A--L and (H,G)-A--L work of Munro and Taussig.

The group of Schwartz et al used the T-lymphocyte proliferative response to study GLPhe responsiveness. They first defined mouse strains with various haplotypes into responders and nonresponders and came up with the same designations as previously assigned by other assay systems. By using F_1 hybrids, the alpha and beta genes inherited from the respective parents are brought together in the trans position i.e. each gene is located on separate chromosomes. In contrast,using recombinant mice, the genetic material is brought together in the cis position i.e. both the alpha and beta genes are located on the same chromosome.

Both Schwartz et al and Dorf et al discovered differences in degrees of responsiveness towards GLPhe between the F_1 hybrids and the recombinant strains of different haplotypes. For instance, a F_1 hybrid derived from crossing haplotypes $H\text{-}2^a$ X $H\text{-}2^b$, such as (B10 X B10.A) F_1, is a high responder just as B10.A(5R) was found to be.

However, the recombinant strains B10.A(2R), B10.A(4R), and B10.A(18R) are nonresponders even though they are derived from the same two nonresponding haplotypes i.e. H-2^a and H-2^b, as the F_1 hybrid (Schwartz et al, 1976). Strains of haplotypes H-2^k and H-2^s, which are nonresponder strains to GLPhe, can also complement each other, but here the cis configuration produces a stronger response than the trans configuration. For instance, (B10.BR X B10.S)F_1 (IA^kIE^s) and (C_3H X SJL)F_1 (IA^kIE^s) are weak responders to GLPhe whereas the recombinant strain B10.HTT (IA^sIE^k) and B10.A(9R) (IA^sIE^d) are high responders (Dorf et al, 1976).

This demonstrates that the degree of responsiveness of F_1 versus recombinants is due to distinct chromosomal relationships of the relevant complementing genes i.e. alpha and beta. It could be possible that all the trans H-2^s configurations are inefficient, or even though they are statisically significant, they are not meaningful biologically. Another possible explanation of the cis phenomena was that it was due to gene dosage. This, however, has proven unlikely since when the (B10.HTT X A.CA)F_1's were tested the alpha allele and the beta allele were dominant in the trans position. This cis effect can still be seen if the (C57Bl/6J X A/J)F_1 is used in transfer experiments instead of in the T cell proliferation assay. The major hypothesis still utilized to explain the cis phenomena is during mammalian evolution it was advantagous to keep the Ir genes together in a group i.e. in the cis configuration. Therefore, cis configurations of the Ir genes are more efficient than trans configurations.

6. Antigen Specificity of Ir Genes

The utilization of the T cell proliferation assay has also allowed for speculation on how the Ir genes maintain good antigen specificity, as well as in which cells both the Ir gene allelles, alpha and beta, are located. By using the classical Farr antigen binding assay, it was demonstrated that an inclusion group for the polypeptides GLT^5, GLT^{15}, and GLPhe exists. In otherwords, the antibody response to these three polypeptides utilize the same Ir genes in their regulation.

Four possible theories of how the Ir genes show precise antigen specificity have been proposed. The first is that there exists a number of Ir loci which pertain to or govern the response to a small set of antigens. The second is that there is a limited number of Ir genes and each Ir allele confers responsiveness to more than one antigen. The third is that there is a requirement for many Ir genes in order to respond to every one antigen. The fourth theory which is accepted as reasonable by most groups is a combination of the above three theories. This theory states that there exists a limited number of Ir genes containing polymorphic alleles and in order to respond to certain antigens, a bigenic control is required (Dorf et al, 1976).

7. Assay Systems for Ir Gene Models

At the present time, studies on Ir gene function with regards

to responsiveness and unresponsiveness is undergoing a change in assay methodology. All of the experimental data cited in this section has been utilizing H-2 congenic mice, chimeric mice, and H-2 mutant mice for in vivo assay systems. A more advantageous in vivo methodology in use presently is transgenic mice.

Many groups have produced transgenic mice by injecting a cloned E_α^k gene into embryos that do not express the E_α gene such as in the $H\text{-}2^{bxs}$ hybrid. It was found that this gene is incorporated into the germline in a normal mendelian manner i.e. the gene is integrated into one autosomal chromosome. LeMeur et al (1985) demonstrated that in $H\text{-}2^s$ and $H\text{-}2^b$ mice, the cause for unresponsiveness to synthetic polymers such as GLPhe was due to a deletion of the promoter and first exon in the E gene. It has been documented that if E_α is not produced, then the already formed E_β remains in the cytoplasm and is not expressed on the cell surface (Steinmetz,M., 1985). With injection of a new E_α gene, a sequence of only 8.2 kb was required to get E_α gene expression in the transgenic mice and this E_α gene expression was tissue specific.

The assays used to demonstrate the effectiveness of this gene transfer or the successfulness of the transgenic mice, were gamma interferon (IFN) responsiveness, monoclonal antibody recognition, and antibody formation. It was discovered that successful transgenic mice responded to gamma interferon in vitro via heightened expression of E_α by their macrophages. By introducing a monoclonal antibody which recognizes $E_\alpha^k/E_\beta^{bors}$ into the assay, it was shown that a structurally complete E complex does exist in these mice. The E complex was deemed

functional since a new immune response or antibody formation occured to antigens such as GLPhe. Antigens like GLPhe require gene complementation to occur between the I-A and I-E subregions of the H-2 complex in order to produce a response. Since an immune response has been elicited, this indicates a functional E complex in cell interactions such as APC - T cell and T cell - B cell interactions.

The only major discrepancy to occur between the groups using transgenic mice in the study of Ir genes, their regulation, and function, has been that usually I-E genes are expressed mainly on B cells that have IgM on their cell surface. As mentioned above, if gamma - IFN is added to cells, an increase in E_{α} gene expression is seen. This increase in E_{α} gene expression via gamma - IFN stimulation is also seen in monocytes in vitro (Yamamura,K. et al, 1985). This group, however, noted a natural elevated constitutive expression of both $E_{\alpha}{}^{d}$ genes and $E_{\beta}{}^{b}$ genes in their transgenic mice. Their transgenic mouse cells did not respond to gamma - IFN as the other aforementioned groups had demonstrated previously. Whether this is a major flaw with methodology or an exception to the rule has yet to be determined.

Overall, the advantages of utilizing transgenic mice over H-2 congenic, H-2 recombinant, and H-2 mutant mice in Ir gene studies far outway the minor disadvantages. Transgenic mice have a transferred, cloned gene that is well characterized and inserted neatly in the mouse's genome. This cloned gene can be modified in vitro via exon shuffling or mutagenesis and, similar to the congenic and recombinant mouse strains, it is possible to breed new mouse strains which carry

the inserted E gene by crossing these transgenic mice with the appropriate congenic inbred or recombinant strain. Therefore, although the "older" in vivo methods are reliable and are still being utilized for the study of Ir gene regulation and function, molecular biology is forging ahead and transgenic mice will become the new wave in Ir gene methodology.

19. Localization and Function of Complementing Ir - GLPhe Genes in Cellular Interactions

The T cell proliferation assay has been useful in the designation of the location of the Ir gene products, alpha and beta, in the GLPhe system. It was found that adding parental T cells primed to GLPhe of alleles alpha$^+$ beta$^-$ or alpha- beta$^+$ to DNP- FGG primed B cells of the (C57Bl/6 X A)F_1 {a responder with alleles beta$^+$alpha$^-$}, no T_H activity was detected. This shows that the alpha and/or beta genes are not expressed exclusively in B cells in order to respond to GLPhe but that in order to get a GLPhe response in the T cell proliferation assay, the Ir genes are required in the T cells (Katz et al, 1976).

Experiments have also been performed that imply that either both Ir genes are expressed in T cells or one or both of the Ir gene products are located in macrophages. This theory was proposed when it was shown that to obtain T cell proliferation, antigen presentation by macrophages is required (Rosenthal et al, 1975). It was also shown that (responder X nonresponder)F_1 T cells are only activated by the responder parent macrophage (Shevach et al, 1973). This will be

discussed in detail later.

With the above conclusions drawn from T cell proliferation studies performed with GLPhe, the system did not follow the outline of the Ir gene regulation of response to (T,G)-A--L as outlined by Munro and Taussig. They proposed that one Ir gene in this system was located in the T cells and the other complementary Ir gene was located in the B cells. They classified nonresponders according to whether a T cell helper factor was produced or not. Therefore, two categories of nonresponders to (T,G)-A--L exist. These are the nonresponders that cannot produce the T helper factor (T_HF) and the nonresponders which can produce the T_HF but their B cells cannot bind or accept this factor. The work done with GLPhe also exemplifies some of the same type of data as the (T,G)-A--L system, however, no work has been done on T_HF production in the GLPhe system.

There are three possible locations of the Ir genes then in keeping with both the GLPhe and (T,G)-A--L systems. These are that in T cell proliferation, both Ir genes are required but only one Ir gene is necessary to produce T_HF. In otherwords, one Ir gene controls the differentiation process of the T cell and therefore T_HF can be produced, while the proliferation of T cells requires the presence of both Ir genes. The second theory is that in order to get T cell proliferation, two cell types are required. These cell types could be two different T cell subsets each with one Ir gene located in each cell. Therefore, animals could be nonresponders phenotypically and yet still produce T_HF. The last possibility is that one Ir gene product is located in the macrophage, and if the data of Munro and

Taussig fits in with the gene complementation observed in the GLPhe system, then this same Ir gene product should also be located in the B cell. The other complementary Ir gene is expressed in the T cell in order to give rise to T_HF. If this third postulate is true, then the responder macrophage would not be necessary for T_HF-producing T cell priming or for the T_HF production with secondary antigen stimulation.

Radiation chimeras have been used to study the presence of the Ir-GLPhe genes and their products in complementation at the cellular level rather than the generic level. Briefly, F_1 hybrids of type ($alpha^+beta^-$ X $alpha^-beta^+$) were irradiated with 850-900 rads. These F_1 hybrids are given bone marrow cells from both parents which have been treated with anti-Thy-1 plus complement. In the following two to twelve months the animals were injected with GLPhe and the subsequent PETLES assay was performed.

Schwartz et al (1979) found no complementation at the cellular level in (2R) → B10 and (18R) → B10.A radiation chimeras using the above protocol. This experiment indicates that for complementation to occur, both Ir - GLPhe gene products should be expressed in the same T cell. Since this statement was proposed on the basis of negative results, control experiments were performed. First it was made sure that the lack of proliferation was not due to a general hyporesponsive immune system. This was tested by producing syngeneic chimeras. For example (5R) → (5R) and {(2R) X B10} F_1 → {(2R) X B10} F_1 radiation chimeras are responder cells mixed with responder cells therefore T cell proliferation was expected in the aforementioned assay and was obtained. The possibility of unbalanced

chimeras was also considered and found not to be a problem by using one antigen under Ir gene control to which one set of parental cells will respond to while the other set of parental cells will not respond. These experiments showed that the chimeras were balanced approximately 50:50 with each of the donor parental cells.

Since the parental cells came from mice of different haplotypes, it was suggested that the nonresponsiveness of the F_1 chimeras to GLPhe was due to a suppressive mixed lymphocyte reaction. This was also proven false by producing responding chimeras. In otherwords, in these chimeras both Ir gene products are in the same cell. In this case, no extraneous mixed lymphocyte reaction was observed and therefore a suppressive MLR suggested as the cause for the nonresponsiveness to GLPhe was incorrect. This does not, however, discredit the possibility that in the chimeras, a collaboration across their I-A barriers is possible.

This group also designed an experiment to demonstrate that in the formation of the F_1 chimeras, the failure to respond to GLPhe was not a failure of cells carrying either $alpha^-beta^+$ or $alpha^+beta^-$ chains to collaborate, but that in order to respond to GLPhe, one cell type from an $alpha^+beta^+$ donor is required. This was done by immunizing an F_1 chimera with DNP - OVA and then treating this recipient with anti-H-2 sera plus complement from one or the other parent. These cells were then tested for responsiveness to DNP - OVA presented by an antigen presenting cell from the opposite parental haplotype. It was found that responsiveness to DNP-OVA occurred.

Schwartz et al also investigated the Ir gene requirements for

antigen presentation. By immunizing (NR X R)F_1 with GLPhe, collecting PETLES plus adding nonimmune GLPhe pulsed spleen cells from either F_1 responders or one or the other parental nonresponders, it was found that both Ir - GLPhe gene products must be located in the same antigen presenting cell in order to stimulate a primed T cell proliferative response to GLPhe. This conclusion was based on the fact that the parental cells failed to present the GLPhe whereas the F_1 cells could present GLPhe. Even a mixture of both parental antigen - presenting cells could not present the GLPhe.

These experiments support the two gene theory of Ir-GLPhe responses which was mentioned earlier. The need for having a responder antigen presenting cell containing both Ir gene products does not support Munro and Taussig's theory for Ir control of (T,G)-A--L. Since both gene products are located on the surface of the antigen presenting cell and that is where they function, the associative recognition type of model for antigen presentation agrees more with the two gene model of Ir - GLPhe control rather than the dual recognition model.

Most groups agree that the alpha and beta chains or gene products are produced in the cytoplasm but the method of surface expression of the two products has not been definitively identified. Warner et al (1977) have proposed that the alpha product allows the beta product to externalize. This occurs either via enzyme modification or via a type of biological transport. This beta gene is a T cell extracellular product thereby allowing the T cell to recognize antigens. This theory is supported by the data provided by

Schwartz et al above, however, the blocking data using anti-Ia sera does not support this model. This is because Warner states that if the alpha chain is already formed, and this chain accounts for the externalization of the beta chain, then no inhibition should be seen with the anti- Ia sera. Inhibition, however,does occur here as shown by Schwartz et al (1976). As mentioned before, Jones et al (1978) also said that the F_1 hybrids and recombinant mice have gene products of I-E and I-A located in the cytoplasm and eventual externalization is prevalent. Blocking studies using anti-Ia antisera did not support this statement although there seems to be a structural difference when looking at the Ir gene products of mice with different haplotypes.

The group of Longo and Schwartz (1980) then examined the requirement of Ir-GLPhe genes in the T lymphocytes. By using a variation in the previous assay of chimera formation, they discovered that there is not a requirement for both Ir genes to be present in the T cell. The assay used is as follows; F_1 chimeras were formed as previously explained and these spleen cells were transferred into acutely irradiated F_1 chimeras. This transfer of spleen cells was the transfer of parental antigen presenting cells, no F_1 antigen presenting cells were present. In addition to the spleen cell transfer into F_1 chimeras, F_1 bone marrow cells that were treated with anti-Thy-1 were also transferred. These cells were the source of F_1 APC. These mice responded to GLPhe and demonstrated that the F_1 APC contained both Ir-GLPne genes and therefore was able to present antigen to the T cells. Since the T cells were a mixture of both

parents, which were nonresponders to GLPhe i.e. alpha$^+$beta$^-$ and alpha$^-$beta$^-$ T cells, then both Ir genes do not need to be present in the T cell in order to respond to GLPhe.

The next process this group decided to examine was the difference in the environments in which the T cells developed. If $(P_1 \times P_2)F_1$ T cells mature in a nonresponder environment i.e. P_1 then they will become nonresponsive to the antigens that P_2 T cells usually respond to. On the otherside, if F_1 T cells develop in P_2 environment then the T cells do not respond to antigens normally responding to in P_1. Therefore, T cells that develop in either parental nonresponder environment are GLPhe unresponsive. Even placing responding F_1 cells into nonresponsive parental environments does not overcome this phenomena. Therefore, T cells must mature in a high responder environment to the particular antigen in order to respond appropriately to the antigen.

To get responsiveness to GLPhe, both T cells, irrelevant of the response status, must develop in a responder environment and at least one cell type expressing both Ir-GLPhe genes is required to be present i.e. APC. Longo and Schwartz propose that complementation occurs at the level of the antigen presenting cell via a post--translational assembly of the two gene products and not by two cells each expressing one responder allele in cooperation. In general, the studies of the genetic control of the immune response indicate that T cells recognize small regions of the Ia antigen molecules unique to particular combinations of the alpha and beta chains.

9. Class II Antigens and Their Relationship with the Ir Genes and Their Products

While alloantisera was used to identify the Ir genes in the I region, the alloantisera also identified a new class of antigens located on B cells, the vast majority of macrophages, depending on their origin, (Hammerling et al 1975, Dorf and Unanue 1977) and on a small population of T cells (reviewed by David,C.S. 1976). These molecules were given the generic name of Ia antigens or I subregion associated antigens since they are coded by I subregion genes. The modern term for Ia antigens is now Class II antigens and these antigens were hypothesized to be the products of the putative Ir genes. The alternative theory is that the two antigens, Ir antigens and Ia antigens, could just be associated with one another. Using genetic studies, biochemical, and functional assays this hypothesis was tested.

The genetic studies utilized congenic inbred and recombinant inbred strains in cross - immunizations of allogeneic lymphoid cell adoptive transfers. These studies could not make a separation between the Ir loci and the Ia loci.

The biochemical studies consisted of 2D-PAGE of non-Idet P-40 extracts and immunoprecipitations of ^{35}S methionine-labeled Ia^+ mouse spleen lymphocytes. These studies showed that one locus which is located in the I-A subregion is more than likely the structural gene for an Ia polypeptide chain. The second locus, which may code for the I-E polypeptide chain, seemed to control

the E_β chain located in the I-A subregion, whether expressed on the cell surface after modification, or whether the molecule would remain in the cytoplasm. Via precipitation with anti-I-E antibodies, the I-A and I-E chains were found to be coexistant in a multisubunit complex in the cell with the I-E chain or E_α controlling the expression of the E chain. The complementation of these two genes and their polypeptide chains was also shown by biochemical assays to occur in either the cis or trans chromosomal position (Jones et al,1978).

Similar conclusions could be indirectly drawn from the functional assays results. Most functional assays were performed testing anti-Ia antisera on an assay which measures exclusively T lymphocyte function. This exclusive assay is the PETLES proliferation assay where PETLES stands for peritoneal exudate T-lymphocyte enriched cells. It was shown that anti - H-2 antisera but not anti-Thy 1 or anti-Ig antisera, could inhibit antigen induced proliferation thereby making this PETLES assay specificity known. It was also shown that anti-Ia antisera could inhibit PETLES proliferation of both Ir gene-controlled T-lymphocyte proliferative responses as well as responses to antigens which were not under Ir - gene control.

Using anti - H-2K and/or anti - H-2D antisera did not inhibit the proliferative response of the PETLES assay to either Ir-gene controlled antigen responses or non-Ir-gene controlled antigens (Schwartz et al,1976). These type of studies were also performed with (responder X nonresponder)F_1 mice. A close phenotypic association between Ia antigens and Ir gene products was demonstrated

and if these two genes coded for independent molecules, then the Ia determinant coded for by either or both of the F_1 parental haplotypes should be able to associate with an individual Ir gene product. This was found not to be the case as the inhibition of the PETLES assay with anti-Ia antisera showed the responses were blocked by one of the parental haplotypes while the responses were not effected by anti-Ia antisera directed towards the other parental haplotype. Therefore, inhibition of proliferation by anti-Ia antisera is haplotype specific and the Ia antigens and Ir gene products are linked phenotypically at the cell surface level. It could now be hypothesized, as mentioned earlier, that the Ia antigens may be determinants on Ir gene products.

Two major models to explain the inhibition of proliferative responses with anti-Ia antisera have been proposed. The first model speculates that the Ir gene products are actually part of the T cell antigen receptor. This could be envisioned by a two domain molecule with the Ir gene product located on the variable domain while the Ia antigenic determinants are located on the constant domain. This would then account for the Ir restriction elements role in Ir gene controlled antibody responses and would still allow for the Ia antigen presence and nonclonal expression on cells.

The second model depicts both the Ir gene products and the Ia antigens as products of a "subregion" of the I region. Therefore, only one gene nonclonally distributed in the cells would control both the Ia antigens and the Ir gene products.

The first model proposed from this work is supported indirectly from the model of both Binz and Wigzell and Eichmann and Rajewsky.

Both of these groups provide evidence of the T cell antigen receptor sharing with the B cell antigen receptors, the variable regions i.e.idiotopes. To put this model together with the models developed by Schwartz et al and Frelinger et al, the two domain Ia-Ir molecule could be synonymous with the light chain of Ig of B cell antigen receptors. In this case, it is a portion of the T cell antigen receptor and could therefore function together or in conjunction with the B cell heavy chain. To merge the second interpretation of Schwartz et al with Binz and Wigzell and Eichman and Rajewsky, the model would depict the single domain Ia-Ir molecule acting as a constant portion. This constant portion would be attached to many different variable regions which are expressed on both T and B cells.

Studies have also shown that the T cells produce different factors which contain Ia antigens (Munro and Taussig,1975). This can also fit into all of the above models for it is possible that when the Ia-positive T cell factor is in membrane-bound form, it may be a part of or act entirely as the T cell antigen receptor.

All of these studies using anti-Ia antisera have one major criticism. This criticism is that anti-Ia antisera could contain antibodies against the putative products of the Ir genes therefore could explain the inhibition of T cell proliferation. However, this criticism has been ruled out in the case of the system using GLPhe and/or cytochrome c as the stimulating antigen for T cell proliferation assays. A monoclonal antibody designated Y - 17 has been produced that is directed against the combinatorial determinant formed by the E_β:E_α or A_e:E_α chains of the E molecule (Lerner et

al,1980). With GLPhe and cytochrome c, this A_e:E_α complex is required as the restriction element for antigen presentation. Therefore, although the Ir gene products and Ia antigens can be distinguished separately by means of function, they are intimately associated phenotypically and share many if not all structural similarities. In otherwords, the Ia antigens are the Ir gene products.

(II) UNRESPONSIVENESS

Exposure to an antigen will not always be followed by an immune response. Responsiveness is a multistep process and a defect at any one stage could result in unresponsiveness by arresting the chain of events. In certain systems such as the synthetic polypeptides GAT and GT, the lack of responsiveness or unresponsiveness to these polymers by certain inbred strains of mice, is due to an active process. This process is the generation of suppressor T cells (T_S) in preference of helper T cells (T_H). This generation of T_S versus the two other possible "unresponsive" mechanisms, that is, antigen recognition defects and T and B cell activation, are viewed as distinct events. This classification into separate mechanisms has been drawn since the mechanisms are not causally related although they are heavily intertwined functionally to one another.

1. GAT and T_S Generation

Unresponsiveness due to the preferential generation of T_S

cells has been studied extensively using the synthetic polypeptide L-glutamic acid^{60}- alanine^{30}-tyrosine^{50} (GAT) as well as with the polypeptide L-glutamic acid^{50}-L-tyrosine^{5} (GT). Inbred strains which are responders to GAT are of the haplotypes a, b, d, and k whereas nonresponders to GAT are of the haplotypes p, q, s, r, w, f, and j (Martin,Maurer and Benacerraf, 1971). It was found that in order to produce anti-GAT antibodies, the presence of thymus-derived helper cells containing the relevant Ir gene was necessary (Kapp, Pierce, and Benacerraf, 1973). Therefore, this group determined that the defect in nonresponders to GAT is due to lack of GAT specific T_H cell function. B cells in both the GAT responders and GAT nonresponders were functional, although the nonresponder B cells tended to be rendered tolerant to GAT easier than the responder B cells. (Benacerraf, Kapp, Pierce, and Katz, 1974).

These groups then examined in more detail the mouse strains for the in vitro culture system for nonresponsiveness to GAT. By a preimmunization dose of GAT, responders to GAT - MBSA became suppressed. The injection of GAT into responders induced T_S cells which were antigen specific (Kapp, Pierce, Schlossman, and Benacerraf, 1974).

2. Phenotyping Suppressors in the GT System and Is Genes

Similar experiments were performed using GT. Here it was found that some nonresponder animals produced an anti- GT antibody response after immunization with GT conjugated to methylated bovine

serum albumin (GT-MBSA) (Debre, Kapp, and Benacerraf, 1975) If a GT preimmunization is given three days before GT-MBSA, the primary anti-GT antibody response is suppressed. However, the secondary response to GT-MBSA remained normal after the GT pre-immunization. This unresponsiveness to GT-MBSA can be transferred into normal syngeneic recipients with spleen cells or thymocytes from GT-primed Balb/c mice. The conclusion was drawn that the administration of GT in Balb/c mice stimulated suppressor cells and therefore Balb/c mice are considered to be phenotypic suppressors to GT. In some inbred nonresponders to GT, suppression to GT-MBSA did not occur utilizing the above protocal. Mice that are nonresponders to GT but are not suppressed by a GT preimmunization are classified phenotypically as "nonsuppressor" strains (Debre, Kapp, Dorf, and Benacerraf, 1975). Therefore, immune suppression does not account for all GT nonresponders.

The two distinct phenotypes of "suppressors" and "nonsuppressors" to GT in nonresponders was shown to be controlled by genes linked to the MHC. These genes were called immune suppression or Is genes since mating of a nonsuppressor to a suppressor strain resulted in a suppressor F_1. This also illustrates that GT specific unresponsiveness is a dominant trait. The Is genes have been demonstrated to be antigen specific since GT doesn't suppress GT-MBSA responses in $H\text{-}2^q$ mice whereas GAT does suppress GAT-MBSA responses in $H\text{-}2^q$ mice i.e. $H\text{-}2^q$ Is genes recognize the antigen GAT not GT.

As with the immune response or Ir genes, GT suppression was also controlled by complementing Is genes. It was demonstrated

that there was a restriction in the ability of GT suppressor genes to complement if they arose from different haplotypes. The genes responsible for control of GT-specific suppression are different in the suppressor haplotypes d, k, and s, therefore Is gene complementation is actually a variation of coupled complementation (Debre, Waltenbaugh, Dorf, and Benacerraf, 1976).

To further examine the question of whether T_S inhibit GT antibody responses in the nonresponder, cyclphosphamide at a concentration of 200 mg./kg. was administered intraperitoneally into mice. Two days later a 100 ug. dose of GT in adjuvant was administered and 10 days later a challenge of 10 ug. GT-MBSA in complete Freund's adjuvant was given intraperitoneally. The lapse of twelve days between the cyclophosphamide and GT-MBSA injection allowed for the recovery of B cells. In this case, no suppression occured by the GT preimmunization probably because the cyclophosphamide had effected the GT-specific T_S cells. The two distinct phenotypes of nonresponders still held in this situation i.e. suppressors and nonsuppressors (Debre, Waltenbaugh, Dorf, and Benacerraf, 1976).

3. Suppressor Cell Generation

It was found that the generation of suppressor cells is not a one cell stage but suppression is mediated by a series of three T cells designated T_S1, T_S2, and T_S3 (Reviewed by Paraskevas, 1984). In between each consecutive T_S generation is a T cell suppressive factor (T_SF). In otherwords, T_S1 produces and releases T_SF_1 which induces T_S2. T_S2 then produces and releases T_SF_2 which induces the

effector cell T_S3. T_S3 has been established as the end stage effector suppressor cell. Of the nonresponder mouse strains to GT, it was found that mice of the "suppressor" phenotype produce T_SF_1 and ultimately generated T_S3 cells (Germaine, 1980). An example of these mice are the (B6 X A/J)F_1 hybrids. However, the nonsuppressor mice were distinguished into two categories:

(1) mice that are unable to produce TsF_1 but can respond to an allogeneic T_SF_1 (eg.) A/J strain

(2) mice that were able to produce T_SF_1 but they could not generate the second order T_S2 (eg.) C57Bl/6J.

As mentioned above, the F_1 hybrids of these two nonsuppressor strains could produce and accept T_SF_1 and were therefore labeled as "suppressors" (Germain, Waltenbaugh, and Benacerraf, 1980).

At a first glance there appears to be an analogy between Ir gene complementation and Is gene complementation. However, this is not the case in the GLPhe system since complementation was demonstrated by the responsiveness of recombinant strains and F_1 hybrids derived from nonresponder strains. In contrast, recombinants between suppressor mouse strains lose their ability to generate suppressor cells in the GT system but still cannot be demonstrated to behave as unequivocal responders. Even up to this date this issue remains unresolved as to the actual interaction structurally and functionally of Ir and Is gene complementation. There is even some

doubts if these two mechanisms even exist at all. The fact still remains, however, that generation of T_S cells does involve many T_SF and T_S "subsets". Each of these T_SF contain separate and distinct properties and therefore are different T_SF factors.

The generation of GT-T_SF_1 occurs across H-2 and I-J barriers and therefore is not H-2 restricted. The GT-T_SF_1 consists of one polypeptide chain containing both the GT binding site and the I-J^+ determinant. No immunoglobulin constant heavy chain region gene determinants have been located on this suppressive factor. This GT-T_SF_1 as mentioned before induces T_S2 and this induction is antigen dependent.

The GT-T_SF_2, like GT-T_SF_1, is antigen specific in its suppressive activity and contains an antigen binding site and I-J^+ determinants. These two sites are present on separate polypeptide chains which are held in close association by a disulfide bond (cysteine-cysteine), however, this association is not a covalent one. Unlike GT-T_SF_1, GT-T_SF_2 is H-2 restricted by the I-J subregion (Kapp, Sorensen, Pierce, 1983).

Although the J molecule has been considered to be involved in T_S induction (Murphy et al, 1976), recent molecular biology evidence denies the existence of the I-J subregion genes between the I-A and I-E subregions (Steinmetz, 1982). The nature of the J molecule still remains unresolved.

Many other mechanisms of T_S induction have been proposed with the synthetic polypeptides as well as other antigens which demonstrate some form of gene complementation. For instance, with regards to

responsiveness to lactate dehydrogenase B (LDH-B), strains which carry the E_B^k allele give no response but instead activate T_S (Baxevanis et al, 1982). Therefore, the E molecule may play a role in T_S cell induction.

It has also been suggested that suppression may be due to presentation by an antigen presenting cell that is different from the one that triggers an immune response. Germain et al (1980) have suggested that the triggering of GT-T_S2 involves cell interactions mediated by GT with either I-A/I-E gene products or I-A/I-C gene products on the cell surface. The cell implied in this interaction is a macrophage, a suppressor macrophage in this case, therefore following a mechanism similar to Ia presentation to T_H cells. This type of antigen presentation and induction parallels Is gene function to Ir gene function in cases such as GLPhe.

There has also been a suggestion that specialized antigen presenting cells exist in the spleen and skin and these are necessary for the activation of effector T_S (Lowy et al, 1983) (Granstein et al, 1984). These antigen presenting cells are distinguished from the other antigen presenting cells by the expression of I-J determinants and their sensitivity to cyclophosphamide (Granstein et al, 1985).

Another approach to unresponsiveness is based on the fact that in a multideterminant antigen, key antigenic determinants regulate the immune response. This fact has led to the view that certain antigen epitopes induce exclusively T_S cells. These "suppressor determinants" have been shown to exist to certain antigens such as different lysozymes i.e. HEL = chicken egg-white lysozyme

(Sercarz et al, 1978).

Therefore, unresponsiveness provides a unique question for the immunologist encompassing genetics, cell-cell interactions, and cellular byproducts or factors. With regard to an organism's ability to delineate self and non-self, the mechanism whereby unresponsiveness occurs will provide an answer not only to demonstrate that unresponsiveness is an important function of the immune system but some light will be shone on the mechanisms of the immune system in general and how the immune system functions.

(III) 6 Hr. Complexes and Antisuppression

Several years ago, the group of Paraskevas et al identified in the serum of mice 3 - 6 hours after immunization a unique and highly immunogenic form of processed antigen. This antigen was complexed with immunoglobulin (Orr et al, 1973) and this combination was simply called "6 hour complexes". Their induction represents a universal phenomenon since they are induced by all forms of immunization: foreign antigens, allogeneic cells and even syngeneic tumor cells (Paraskevas.F. and S.T. Lee,1976).

There are several reasons to believe that the " 6 hour complexes" do not represent conventional antigen - antibody complexes, not only because they first appear in the blood within 3 hours after immunization (peaked at 6 hours), but more importantly because their formation depends on a factor which can be induced in vivo and in vitro (a lymphokine) (Paraskevas, F. et al, 1984).

The factor is produced in vivo by Ly 1^+ T cells and has tentatively been called Ig and antigen complexing factor or IACF. IACF formation depends on a macrophage - T cell interaction and is composed of Ia antigen and idiotypes which are not covalently linked.

The unique property of the complexes is the fact that they are strongly cytophilic for T cells although B cells and macrophages may also take up the complexes. The types of antigens which do not generate these "6 hour complexes" are nonimmunogenic proteins like gelatin or syngeneic cells i.e. autoantigens. There is also a minimal requirement for the production of complexes against synthetic polypeptides. This requirement stipulates that the polypeptide consists of a minimum of three amino acids (Paraskevas et al, 1984).

As mentioned above, the " 6 hour complexes" are cytophilic for a subpopulation of T cells which are $Ly2^+$ Ia^+ F_cR^+. In contrast to conventional antigen - antibody complexes which bind to T cells through F_c receptors, the binding of " 6 hour complexes" depends primarily if not exclusively on the Ia antigens. Serological evidence suggests that the Ig in the " 6 hour complexes" possesses anti-idiotypic specificity (Paraskevas et al, 1984).

Since the induction of the complexes, their early appearance after immunization, and the consistency of their structure and composition is universal to all antigens (excluding the aforementioned exceptions) , the complex formation represents a fundamental form of immune response to antigens with biological significance. The biological significance of these 6 hour complexes is that T cells which have taken up these complexes result in a marked enhancement of

antibody production (Paraskevas et al, 1976). In addition, the complexes themselves when injected into normal animals enhance the IgG response to a subimmunogenic stimulation up to 50 fold therefore acting as a potent enhancing factor (Paraskevas et al, 1981) (Lee et al, 1981).

The "6 hour complexes" which are induced by allogeneic stimulation markedly enhance the CTL response in vivo due to interference with suppressor T cell function (Paraskevas et al, 1981). Evidence indicates that "6 hour complexes" represent a mediator of an immunoregulatory pathway which has been called antisuppression (Paraskevas et al, 1984).

Antisuppression is the earliest in vivo documented response to antigen. Interference with the activation of antisuppression leads to development of suppressor cells. This has been so far documented in two different models. Injections in vivo of anti-IA^d antibody in Balb/c mice eliminates the formation of the "6 hour complexes" and allows otherwise strong immunogenic stimuli to give rise to the generation of Ly 2^+ suppressor T cells. The relevant haplotype anti I-A antisera suppressed IgG antibody production in the strains tested whereas antibodies to Ia.7, a major public specificity of $E_\alpha{}^k$, had no effect on complex production (Paraskevas et al, 1985). The second model demonstrates that during acute GVH reactions which are known to be associated with profound immunosuppression, the anti-suppressor pathway (induction and effector stages) are abolished.

Antisuppression, as evidence indicates, plays a pivotal role in both humoral and cell mediated immunity. It represents a "master

switch" which determines betweeen immunity i.e. responsiveness and suppression. Unresponsiveness in these terms is due to the interference with antisuppression. This interference could arise from either lack of formation of the complexes or blockage of the complexes function i.e. no cellular uptake or if there is uptake, no activation of the antisuppressor mechanism. Therefore early induction of antisuppression will prevent the premature development of T_S and thus will ensure the generation of the effector mechanisms of immunity. On the other hand, blocking of complex formation or interference with complex function, genetic or otherwise, will lead to the development of T_S and unresponsiveness.

Ir gene complementation may be explained within the antisuppressor framework. 6 hour complex production is dependent on a gene located within the I-A subregion (Paraskevas et al, 1985) i.e. this is where the induction stage occurs. Within the I-A subregion is also where the beta gene for complementation exists (Dorf et al, 1975 and Dorf and Benacerraf, 1975). The second stage of antisuppression or the activation of antisuppression is controlled by a gene located in the I-E subregion i.e. where the alpha gene of complementation exists. It was found that in responders both stages of antisuppression were intact i.e. complex formation and activation of the T cell that takes up the complex. In the nonresponder, unresponsiveness can be shown to be due to defects in the antisuppressor pathway in either the induction and/or effector stage. Therefore, only one set of genes need be implied in responsiveness / unresponsiveness instead of two sets (Ir and Is genes) as suggested by

earlier work (Debre, P., 1976). So two complementing genes determine an active antisuppression while in the absence of either one, antisuppression is defective leading to T_S induction and unresponsiveness.

In this thesis, the main objectives were to study the responses of inbred and inbred congenic mice to the terpolymer GLPhe. A method of analysis to distinguish between responders and nonresponders was developed (a modified ELISA assay). This technique which was found to be very sensitive detects anti - GLPhe antibodies. By attaching GLPhe to a carrier molecule (CGG), the gene complementation requirement could be bypassed. This was useful to attempt to determine whether unresponsiveness was due to the induction of suppression. This was attempted by two methods: (1) pre-immunization with GLPhe before GLPhe-CGG immunization and (2) administration of cyclophosphamide. To determine whether 6 hour complexes were formed in the mouse strains studied, RICA analysis was performed. The transfer of 6 hour serum both syngeneically and allogeneically was performed to determine the functional capacity of the complex in both induction and effector stages.

MATERIALS AND METHODS

(I) Animals

Inbred and congenic mice, six to eight weeks old, were purchased from Jackson Laboratory, Bar Harbor,Maine. These include C57Bl/6J, C3H/Hej, SJL, B10, B10.A, and B10.A(5R). Balb/c mice were obtained from the University of Manitoba Campus Animal Facilities. The abbreviation B10, B10.A, and B10.A(5R) are used for C57Bl/10 SnJ, B10.A/SgSnJ and B10.A/SgSnJ(5R) mice respectively.

(II) Antigens

The random linear terpolymer GLPhe (L-glutamic acid^{56}, L-lysine^{35}, L-phenylalanine^{9}HCl) was obtained from Miles-Yeda Ltd., Israel. The GLPhe was dissolved in saline pH 7.4 before use.

Ferritin (Fe) was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Bovine serum albumin (BSA) was purchased from Miles Laboratories, Elkhardt, Indiana.

Chicken gamma globulin (CGG) was isolated from chicken serum purchased from Gibco Laboratories, Burlington, Ontario. (see isolation for details)

(III) Reagents

Reagents utilized in the following experiments include Freunds' complete adjuvant (FCA) purchased from Difco Laboratories , Detroit, Michigan. FCA was utilized in most of the immunization protocols which required intraperitoneal injections.

P - nitrophenyl phosphate was used as the substrate for color development (Sigma 104 Phosphatase substrate tablets, St. Louis, Missouri). These tablets were made into a workable assay solution by dissolving in 10% diethanolamine (Aldrich Chemical Co. Inc., Milwaukee, USA).

Cyclophosphamide or cytoxan was purchased from Bristol Laboratories, Belleville, Ontario and was dissolved in distilled water to the appropriate concentration required for injection.

For the iodinations, NaI^{125} was purchased from New England Nuclear Corporation, Boston, Massachusetts at a concentration of 100 mCi/ml.

(IV) Antisera

Anti - BSA and anti - Fe were prepared by immunizing mice (Balb/c) with 1 mg. of antigen in FCA two times at weekly intervals and subsequently bled via a heart puncture a week later. The antisera were tested on Ouchterlony to form a line with the appropriate antigen. Goat anti - mouse alkaline phosphatase utilized in the Elisa

assay was purchased from Sigma, St. Louis, Missouri.

(V) Isolation of Chicken Gamma - Globulin (CGG)

CGG was isolated from chicken serum (Mycoplasma tested) from Gibco Labs, Chagrin Falls, Ohio via the ammonium sulfate precipitation method described in "Methods in Immunology" 3rd ed. Garvey, Geser, Sussdorf, (1977).

Briefly, to 50 ml. of chicken serum, a total of 25 ml saturated ammonium sulfate pH 7.8, (by addition of 2N NaOH), was added dropwise to achieve a 1/3 saturation level. Each drop of saturated $(NH_4)_2SO_4$ was not added until all the precipitation from the previous drop had dissolved. At the end of the total 25 ml. addition of saturated-$(NH_4)_2SO_4$, an amount of precipitation will persist. To avoid trapping of serum components, other than gamma-globulin, the mixture was kept stirring for an additional 2 - 3 hours.

A 30 minute centrifugation was performed at 1400g at room temperature. Supernatent was discarded and the resulting precipitated pellet was resuspended in approximately 50 ml. of saline, i.e. up to the original serum sample volume. This pellet contained small quantities of albumin and other serum globulins as well as the majority of gamma globulin found in the chicken serum.

The above precipitation procedure was repeated two more times. On the last precipitation step, the pelleted precipitate was resuspended and dissolved in borate-buffered saline. The volume used for resuspension was approximately half or less than half of the

original 50 ml. serum sample.

The ammonium sulfate trapped within the precipitated gamma globulin was disposed of by dialysis against borate buffered saline for 2 - 3 days at 4°C with frequent changes of buffer. The final dialysis was performed in PBS pH 7.2 and this dialyzed sample was centrifuged at 8000-10,000 rpm at 4°C for 30 minutes to get rid of the aggregates.

Total amount of CGG in solution was quantitated via spectrophotometry. A total of 930 mg. total gamma globulin was recovered and the sample was concentrated to 100 mg./ml. and stored at -20°C.

(VI) Conjugation of GLPhe - CGG

The conjugation of GLPhe to CGG was performed according to the method described by Cheung et al (1977). The starting solution contained 125 mg. GLPhe and 500 mg. CGG in 12.5 ml. of 0.1M sodium citrate. An equal volume of sodium periodate in distilled water was slowly added (total volume = 25 ml.). This solution was kept stirring at room temperature for 2 hours and then dialyzed in 2 litres of 0.05M citrate buffer pH 5.6 for 4 hours. This dialysis was followed by another dialysis procedure in 0.01M sodium carbonate pH 9.5 at 4°C overnight. The solution was removed from the dialysis tubing and stirred for another 2 hours at room temperature. Three aliquots each containing 62.5 mg. of sodium borohydrate in a volume of approximately 5.0 ml.were added over a period of 2 hours. Excessive foaming was

prevented by addition of a drop of octanol. The stirring was allowed for another 2 hours at room temperature and then was dialyzed extensively (4 litres PBS each day for a total of 6 days) at 4°C. The conjugated GLPhe-CGG was precipitated with saturated ammonium sulfate at 45% saturation and centrifuged at 15,000 rpm for 20 minutes. The precipitate was redissolved in 40 ml. of PBS and extensively dialyzed (4 litres PBS each day for 7 days) at 4°C. The quanitity of GLPhe-CGG was determined via spectrophotometry. A total of 121 mg. of GLPhe-CGG conjugate was recovered and stored in 2 mg./ml. aliquots at -22°C until use.

The GLPhe-CGG conjugate was also tested on Ouchterlony gel diffusion against mouse anti - GLPhe and rabbit anti - CGG antisera for the presence of GLPhe and CGG in the conjugate.

(VII) Preparation of Six - Hour Sera (6HS) and Normal Mouse Sera(NMS)

Serum was collected from mice six hours after an intraperitoneal immunization with 100 ug. GLPhe in 0.2 ml. of a saline/FCA emulsion (1:1 ratio by volume). Serum was collected six hours later, via chloroform anasthetization of mice followed by cardiac puncture bleeding. Blood was allowed to clot for approximately 30 minutes at room temperature and the serum was collected. Normal mouse serum was obtained from non- immunized animals in a similar manner and served as controls.

(VIII) Immunization Procedure for Anti - GLPhe Response

Each inbred strain of mice (C_3H, SJL, C57Bl/6, and Balb/c) were divided into two experimental groups consisting of 3 - 6 mice per group. One group was immunized with either 100 ug. or 250 ug. of GLPhe. Another group was immunized with 100 ug. or 250 ug. of GLPhe-CGG. All immunizations were carried out intraperitoneally (i.p.) in a total volume of 0.2 ml. emulsified in a ratio of 1:1 with FCA containing 0.5 mg./ml. of Mycobacterium tuberculosis.

The congenic strains B10, B10.A, and B10.A(5R) were also divided into two groups as described above and immunized with either 100 ug. or 250 ug. of GLPhe or GLPhe-CGG. The only difference between immunization protocols between the inbred mouse strains and the congenic strains was that the immunizing emulsions in the congenic strains was made with FCA containing 1.0 mg./ml. of Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI.)

All mice were bled at weekly intervals up to eight weeks through the tail vein. The serum samples were collected by centrifugation in capillary tubes and were stored at -22°C.

(IX) Immunization Procedure for the Induction of Low Dose Enhancement

When the injection of 6HS was required, the volume of injection was always 0.3 ml. (i.v.). Two hours later 50 ug. GLPhe in saline was

administered in the same mouse i.v. through the tail vein. NMS, injected by this same protocol, served as the control. Animals were bled through their tail veins at weekly intervals up to six weeks. All serum samples were collected by centrifugation in capillary tubes and were stored at -22°C.

(X) PROCEDURE FOR INDUCTION OF SUPPRESSION TO GLPhe - CGG IN NON - RESPONDER MICE

1. Induction of Suppression to the GLPhe Response

Non - responder strains of mice (C_3H/Hej, C57Bl/6J) to GLPhe were preimmunized intravenously (i.v.) with 100 ug. of GLPhe followed two weeks later (in some cases GLPhe was injected i.v. at both two and four weeks) a challenge of 100 ug. GLPhe - CGG in FCA (i.p.).

Mice received 100 ug. GLPhe (i.v.) and were challenged two weeks later with 100 ug. of BSA in FCA (i.p.) and the mice that received only 100 ug. of BSA in FCA (i.p.) served as the specificity controls.

Mice were bled through the tail veins after immunization at weekly intervals for up to six weeks. All the serum samples were collected by centrifugation in capillary tubes and were stored at -22°C.

2. Treatment of Nonresponder Mice With Cyclophosphamide

The design of the experiments for detecting the immune response to GLPhe in nonresponder mice are two fold:

(1) Nonresponders were injected with a low dose of cyclophosphamide (100 mg./kg. body weight) intraperitoneally (i.p.), followed 2 days later with a challenge of 100 ug. GLPhe (i.v.). The response to GLPhe was followed at weekly intervals for up to six weeks.

(2) Nonresponder mice were injected with cyclophosphamide (100 mg./kg. body wt.) ip, two days later,they were injected i.v. with 100 ug. GLPhe and two weeks later they were challenged with 100 ug. GLPhe--CGG. The response to GLPhe was followed weekly up to six weeks.

(XI) Reverse Immune Cytoadherence Technique (RICA)

Details of this technique have been published.(Paraskevas, Lee, and Israels 1971a J. Immunol. 106:160. and Paraskevas, Lee, Orr and Israels 1971b, J. Immunol. Methods 1:17). This method utilizes a 5S hybrid antibody with one anti - Ig site and a second site which is usually specific for a protein such as BSA. Through the first site, the 5S hybrid antibody binds to Ig^+ cells while the latter site attracts BSA - coated SRBC thus forming a rosette.

1. Detection of Ag - Ig Complex in the Six Hour Serum by RICA

Serum from mice 6 hours after immunization contains an Ag - Ig complex which is cytophilic for a subpopulation of T cells in the spleens of mice (Orr - Paraskevas. J. Immunol. 110:456, 1973). T cells in the spleens of mice are normally Ig^-. After taking up the cytophilic Ag - Ig complex from the 6HS, these cells become Ig^+ as detected by RICA. The increase in Ig^+ cells in the spleen cell after incubating with 6HS indicates the presence of the cytophillic complex.

5 X 10^6 spleen cells in Hanks' solution are incubated with 0.2 ml. 6HS at 37°C for 30 minutes in a total volume of 1 ml. Cells were then washed 3 times with Hanks' solution at 4°C. To 1 X 10^6 treated cells in a volume of 0.1 ml., 30 ul. of hybrid antibody and 30 ul. of BSA - coated SRBC were added. The incubation was then carried at 4°C overnight. Rosette - forming cells (RFC) were then counted under a phase contrast microscope in a Bellco welled - slide (Bellco, N.J.) A RFC was identified when at least 4 SRBC were found around a single lymphocyte. Spleen cells incubated with normal mouse serum (NMS) as described above served as the control.

(XII) Method for Detection of Anti - GLPhe Antibody Response

The ELISA Technique

A variation of an enzyme-linked immunoabsorbant assay (ELISA) was used for the detection of the humoral response to GLPhe in the mouse. This Elisa technique was adapted from the Elisa technique

described by Voller et al (Voller,A., Bidwell,D., & A. Bartlett in "Manual of Clinical Immunology",N. Rose & H. Freedman, Eds. p. 506 Amer. Soc. Microbial. Washington, D.C. USA.) The 2 layer antibody technique was adopted. The first layer was the serum bled from mice injected with GLPhe or GLPhe-CGG. The second layer in the sandwich technique is a goat anti-mouse Ig (whole molecule) antibody conjugated to alkaline phosphatase.

Linbro 96 flat bottom well microtitration plates (Flow Lab, McLean, Virginia Cat. # 76-381-04) were coated with GLPhe at a concentration of 100 ug./ml. for 4 hours at 37°C. The excess liquid was removed and a wash with PBS-Tween was performed. Following the wash, a 1% gelatin solution was added to all wells of the plate in order to block the uncombined reactive sites at 37°C overnight. The gelatin was aspirated and multiple washings of the plate with PBS--Tween were performed. The first layer of test antibody at the appropriate dilutions were added to the wells for 4 hours at 37°C. This first antibody layer was then aspirated and the wells of the plate were washed thoroughly. The second antibody layer, goat anti-mouse Ig - alkaline phosphatase, was then added to the wells at the suggested working dilution of 1:1000. This reaction was allowed to carry on overnight at 4°C followed by the reaction with the substrate p - nitrophenyl phosphate in 10% diethanolamine. The reaction was terminated by the addition of 3N NaOH and the plates were read by the Multiskan at 405 nm.

(XIII) Iodination

NaI^{125} was obtained at a concentration of 100 mCi/ml (New England Nuclear Corp., Boston, Mass.). The volume obtained was 50 ul. containing 5 mCi, therefore a dilution was made with NaOH resulting in 5 mCi in 250 ul of NaOH as the stock solution.

The procedure for iodination is the same as described in "Methods in Immunology" 3rd ed. Garvey, Geser, and Sussdorf (1977). 25 ul of the NaI^{125} in NaOH stock solution was added to 75 ul of PBS pH 7.4. 1200 ug. of mouse IgG in a volume of 100 ul. was added to the above solution yielding a total volume of 200 ul. Chloramine T made up to a concentration of 5 mg./ml. in 0.14M PBS pH 7.4 was used as the oxidizing agent. For the volumes used in this iodination, 40 ul (200 ug) chloramine-T was added to the protein and a reaction time of 2 minutes was allowed to proceed. To stop the oxidization reaction and stabilize the iodine groups now added onto the IgG, a solution of 1.2 mg/ml sodium metabisulfate in 0.14M PBS pH 7.4 and a solution of 2.0 mg/ml NaI in 0.14M PBS pH 7.4 were used.

First, 60 ul. of the sodium metabisulfate (250 ug.) was added to the reaction mixture and then 100 ul. (200 ug.) of NaI was added. The final total volume was 400 ul. This solution was put into dialysis tubing and the volume was made up to approximately 1.0 ml. by addition of 0.14M PBS pH 7.4.

This solution was dialyzed in 2 litres of 0.14M PBS pH 7.4 and was changed approximately every 4 - 6 hours for a total of four

changes. At each change of buffer, a sample was taken and counted in the gamma counter until no free I^{125} could be detected in the dialysing buffer. The sample was then collected and stored at 4°C.

A trichloroacetic acid (TCA) precipitation was performed on the iodinated IgG sample as described in "Methods in Immunology" 3rd ed. Garvey, Geser, and Sussdorf, (1977). 100 ul. of the stock $^{125}I^{-}$IgG was added to 900 ul of 0.14M PBS pH 7.4. This solution was then counted in the gamma counter. 50 ul of the above diluted stock solution was placed in a microfuge tube together with 50 ul of a 1% BSA in 0.14M PBS pH 7.4 and vortexed. 100 ul of 20% TCA was then added, vortexed, and the sample was allowed to precipitate at 4°C overnight. The sample was centrifuged at 1500 rpm for 30 minutes at 4°C and the supernatent was pipetted off and separated from the precipitate. 10 ul of the supernatant and the precipitate were then counted in the gamma counter resulting in all the counts being present in the precipitated fraction i.e. the protein or IgG antibody contained all of the label, all the free iodide was removed. Thus, the radioactivity in terms of cpm of the labelled IgG could be correlated to the actual amount of IgG as determined by spectrophotometry.

(XIV) Standard Curve

Mouse I^{125} - IgG was used for the construction of a standard curve. 50 ul. of I^{125} - IgG containing different quantities of IgG (ranging from 3 ng.-> 0.001 ng. as measured by spectrophotometry)

were added to each well of a soft plate (Linbro 96 well flat bottom flexible plates, cat. # 76-374-05, Flow Labs, McLean, Virginia). Each concentration of labeled IgG was plated for a total of eight trials.

After a 4 hour incubation at 37°C, the plate was aspirated and washed once with PBS-Tween (200 ul each well). The plate was then blocked with 1% gelatin solution, 200 ul each well, and incubated at 37°C overnight. The following day the plate was aspirated and washed 6 times with PBS-Tween. The plate was then reacted with goat anti--mouse Ig - alkaline phosphatase for 4 hours at 4°C and then washed 6 times with PBS-Tween. The substrate p - nitrophenyl phosphate in 10% diethanolamine was used for the color development and the O.D. was read and recorded using the Multiskan.

The liquid in the wells of the soft plate were aspirated, the wells were cut out and the presence of ^{125}I - IgG in terms of cpm/well was recorded. These counts indicated the quantity of I^{125} - IgG which actually coated onto each individual well. When these counts were compared to the original stock solution in terms of cpm and actual quantity, the quantity of I^{125} - IgG in each well could be calculated.

When the quantity of IgG in ng/well was plotted against the O.D. reading in each well, a straight line was obtained. Thus IgG antibody response as determined by the ELISA technique in any subsequent experiment can be quantitatively extrapolated from the curve.

RESULTS

(I) Standard Curve

All of the experimental results utilizing the Elisa technique have been presented in one of two forms:

(1) O.D. readings at 405 nm.

(2) ug./ml. quantities of antibody

The ug./ml. measurement is the result obtained from the construction of the standard curve described in materials and methods. The standard curve obtained is shown in Figure 1. A straight line was produced until O.D. readings greater than 1.5 were reached. The Multiskan instrument has a maximum capacity of 2.0 O.D. units and greater than 1.5 O.D. resulted in a deviation from a straight line. Readings greater than 1.5 O.D. resulted in antibody quantities greater than $.162 \times 10^{-4}$ ug./ml. By extrapolation from this standard curve, the O.D. measurements from the Elisa technique can quantitatively be expressed as ug./ml. of anti - GLPhe antibody.

(II) Utilization of Elisa Technique for Tracing Anti-GLPhe Antibody Responses

The progression and development of anti - GLPhe antibody responses were traced in both inbred and inbred congenic mouse strains utilizing the Elisa technique. Balb/c, a responder, C_3H/Hej, C57Bl, and SJL (all nonresponders) inbred mice (Dorf and Benacerraf,

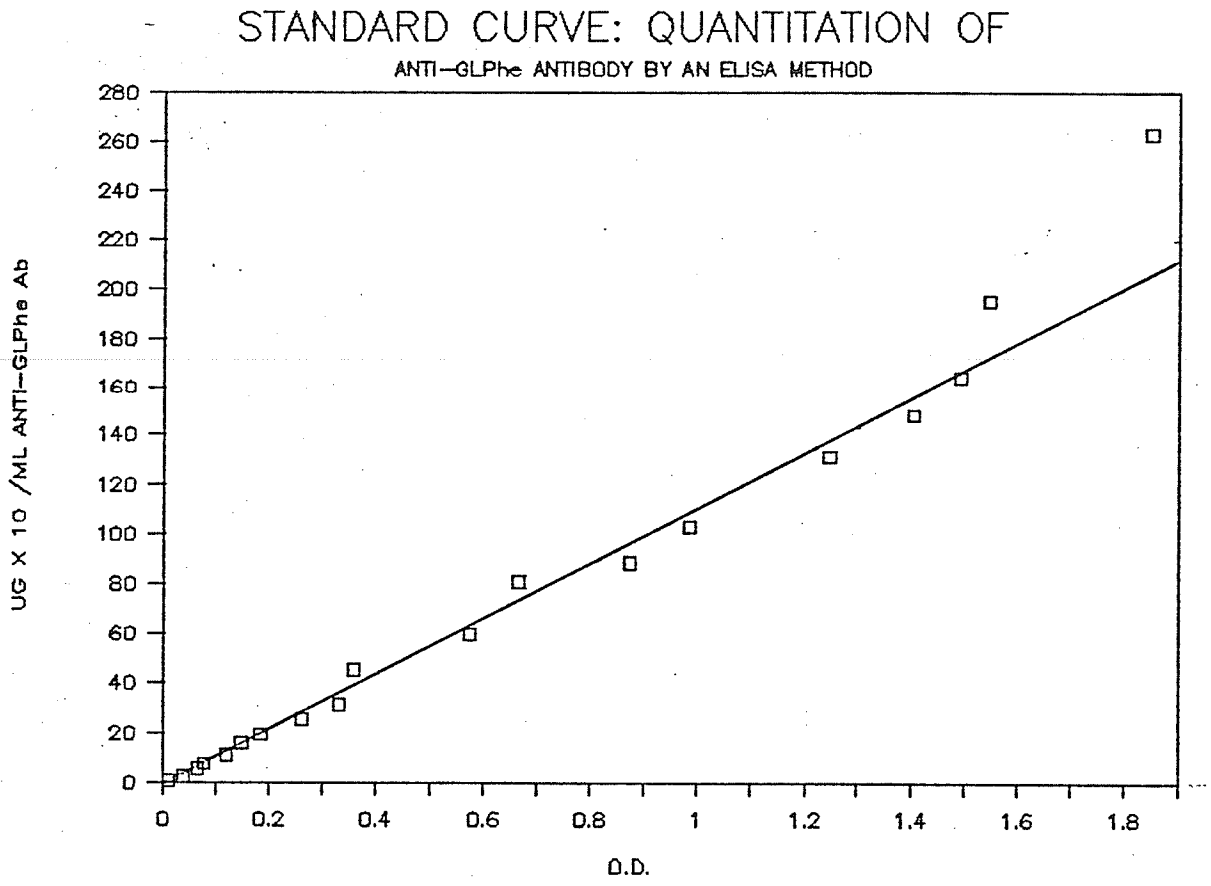
STANDARD CURVE: QUANTITATION OF
ANTI-GLPhe ANTIBODY BY AN ELISA METHOD
UG X 10 /ML ANTI-GLPhe Ab
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
O.D.

FIGURE 1

1975) were screened from day 0 to day 56 after a 100 ug. GLPhe in FCA injection intraperitoneally. Figures 2a, 2b, and 2c presents three dilutions of the serum bled at weekly intervals for up to six weeks. A substantial amount of anti-GLPhe antibody was produced by the Balb/c responder as compared to the nonresponding strains of C57Bl, C_3H/Hej, and SJL as measured in O.D. at 405 nm.

The amounts of the antibody were also quantitated. In other-words, the O.D. measurements obtained from the Elisa assay were converted to ug. of anti - GLPhe antibody per ml. of serum by extrapolating the O.D. measurement on the standard curve described in the materials and methods. All graphs have accounted for background color development due to the technique itself as well as controlling for the color development due to NMS background (Figure 3).

Figure 4 demonstrates that the O.D. measurements derived from the Elisa assays are actual indications of GLPhe antibodies. Balb/c mice hyperimmunized with 100 ug. Fe/FCA (i.p.) or 100 ug. BSA/FCA (i.p.) were tested by the Elisa technique and as shown in Fig. 4, negligible O.D. readings were obtained as compared to Balb/c mice immunized with 100 ug. GLPhe/FCA (i.p.). A control of each mouse strain's normal serum was used during each Elisa assay and as mentioned above, these measurements have been accounted for in the presentation of the data by running controls and standards of NMS from each particular strain as well as running wells with no serum i.e. for background color development.

The inbred congenic strains of B10, B10.A, and B10.A(5R) were also followed from day 0 to day 42 following a 100 ug. GLPhe/FCA

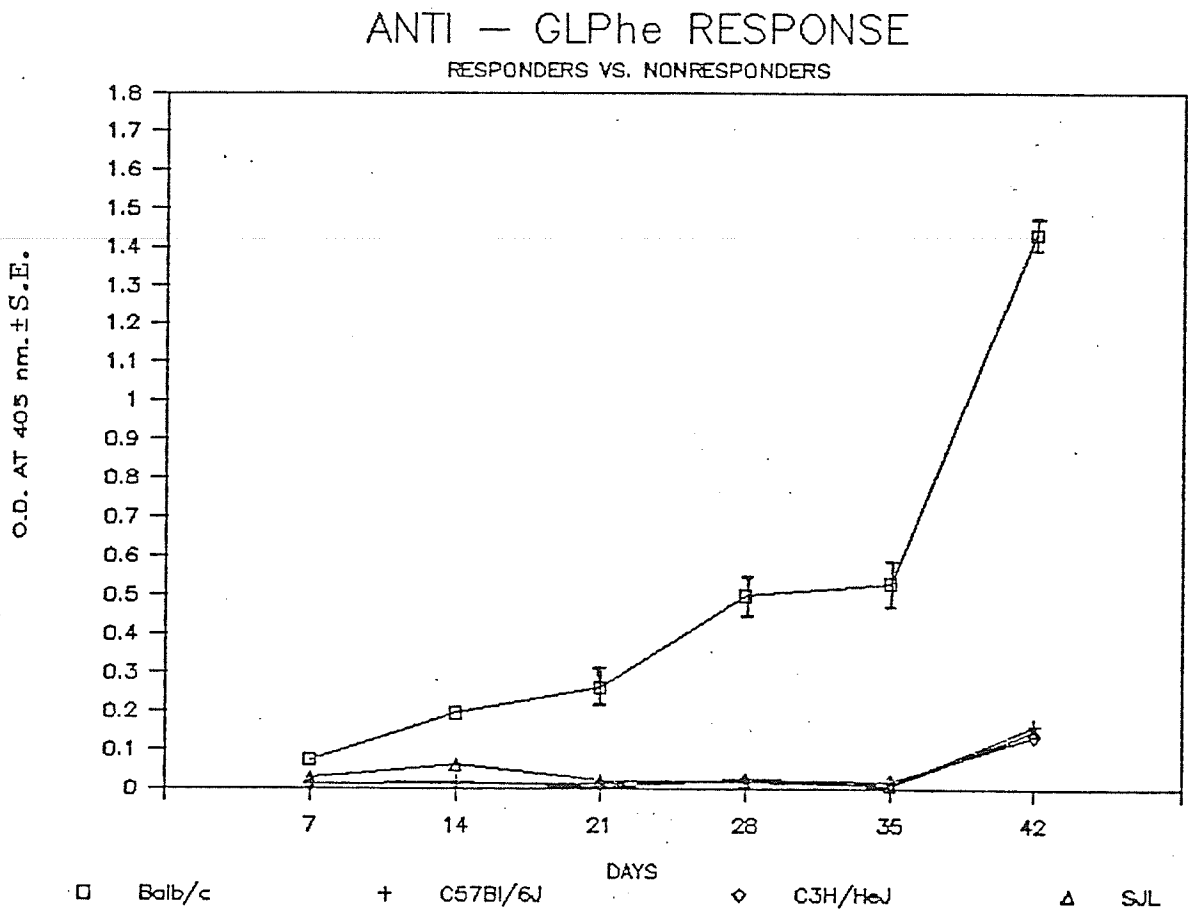

FIGURE 2a: 1/50 dilution of antisera

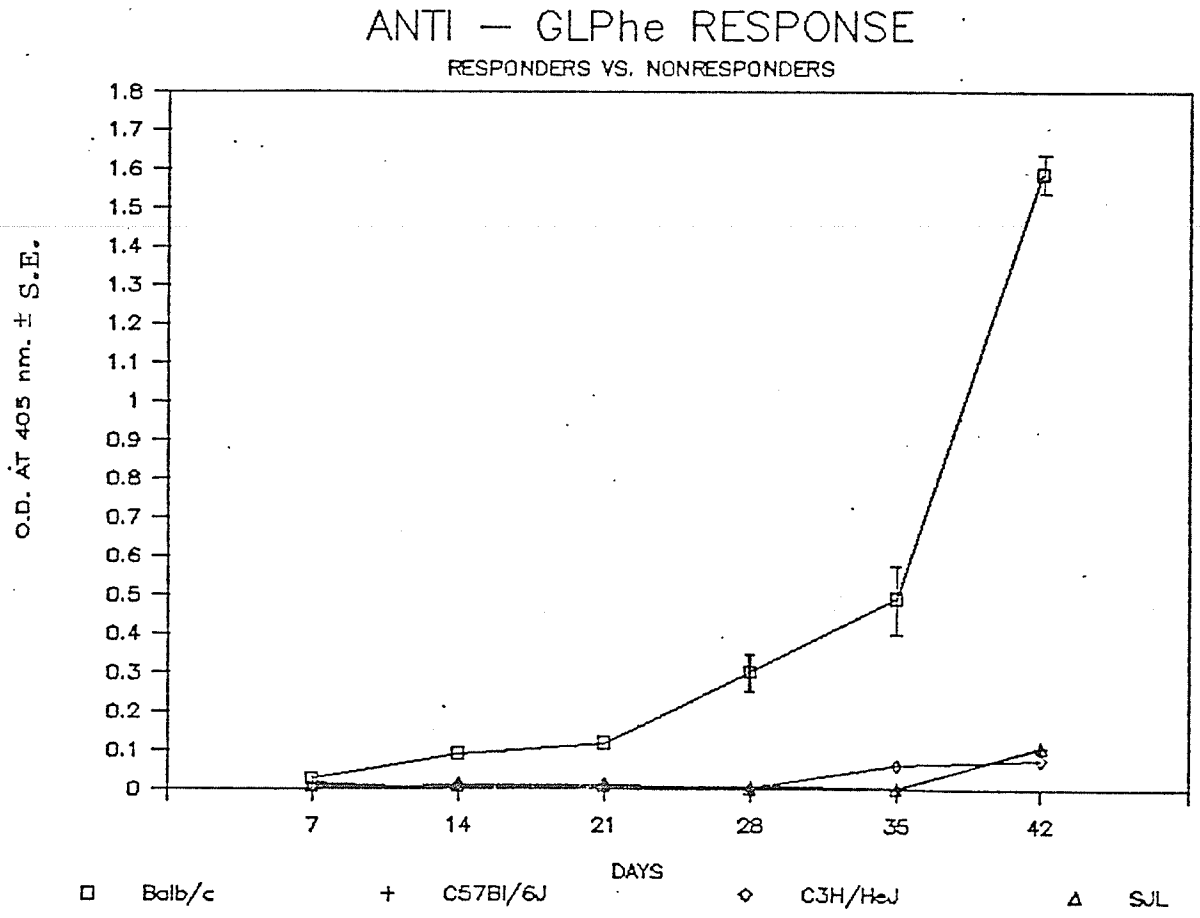

FIGURE 2b: 1/500 dilution of antisera

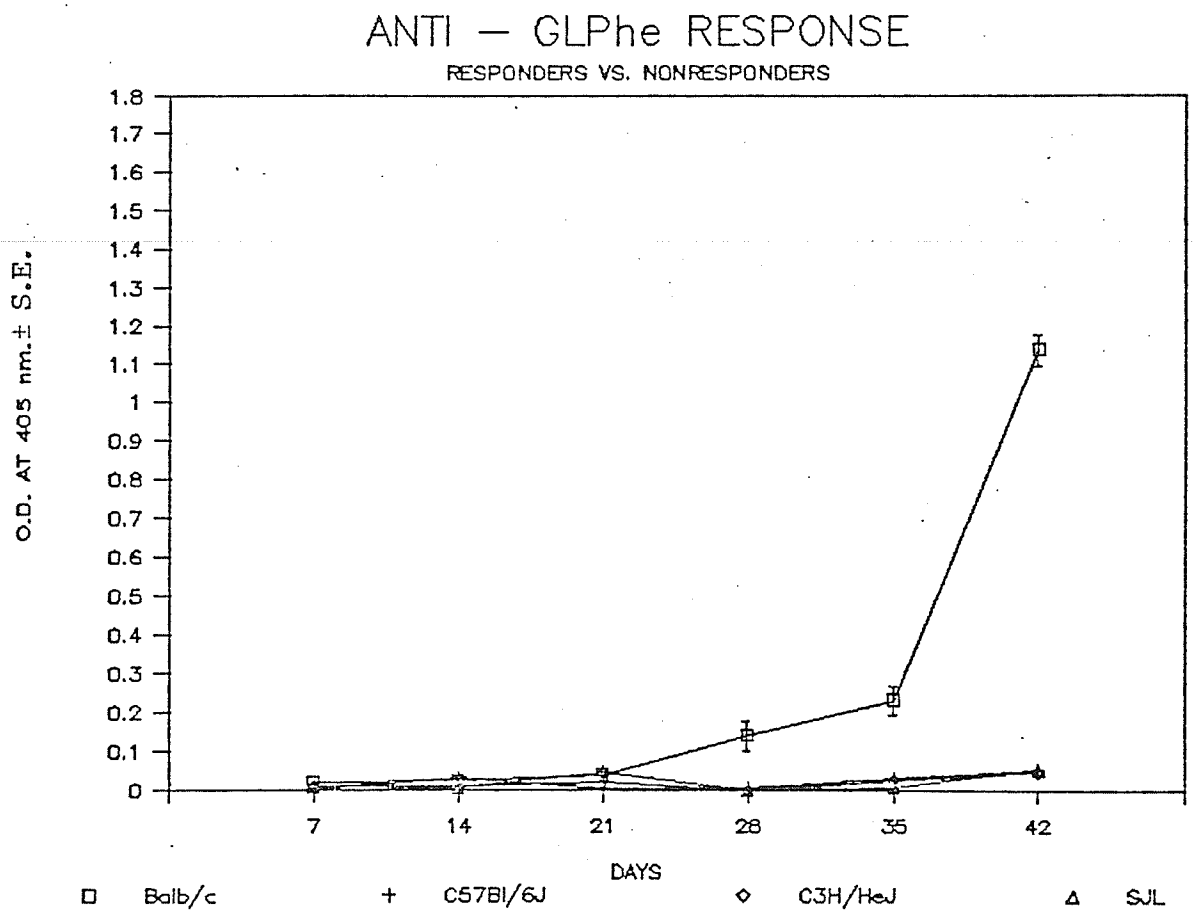

FIGURE 2c: 1/5000 dilution of antisera

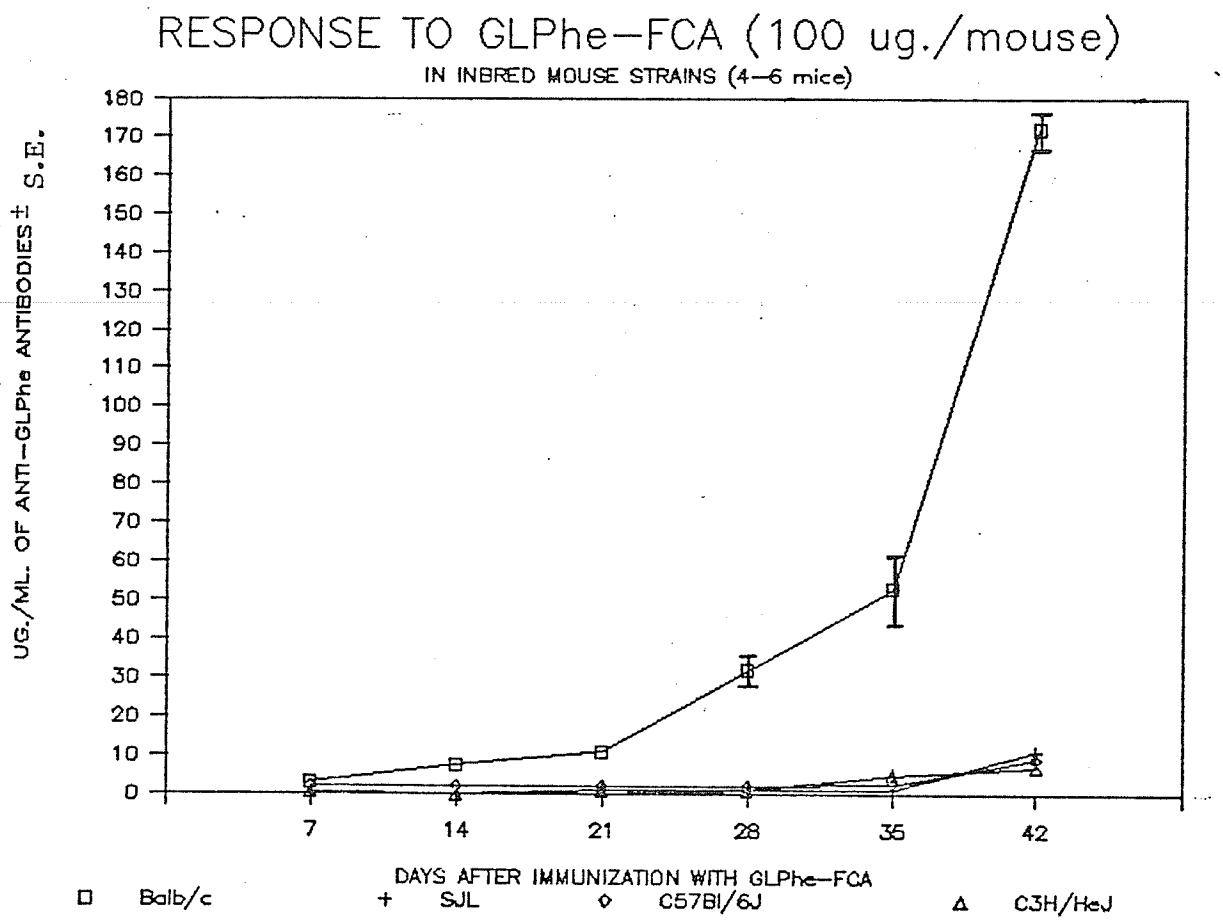
RESPONSE TO GLPhe—FCA (100 ug./mouse)
IN INBRED MOUSE STRAINS (4—6 mice)
UG./ML. OF ANTI—GLPhe ANTIBODIES ± S.E.
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
7
14
21
28
35
42
DAYS AFTER IMMUNIZATION WITH GLPhe—FCA
□ Balb/c
+ SJL
◇ C57Bl/6J
△ C3H/HeJ

FIGURE 3

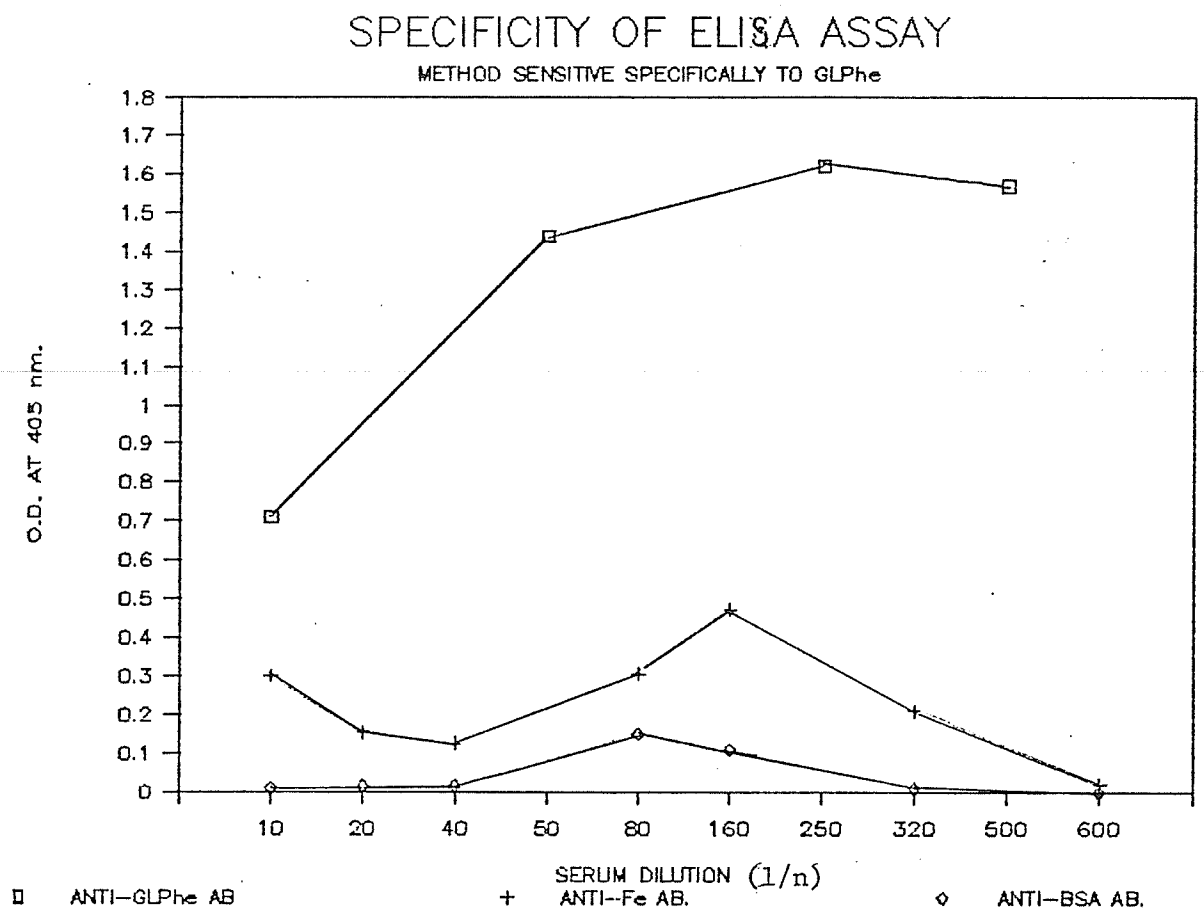
SPECIFICITY OF ELISA ASSAY
METHOD SENSITIVE SPECIFICALLY TO GLPhe
O.D. AT 405 nm.
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
40
50
80
160
250
320
500
600
SERUM DILUTION (1/n)
ANTI-GLPhe AB
ANTI--Fe AB.
ANTI-BSA AB.

FIGURE 4

(i.p.) injection. The Elisa method has been utilized in this case to define responders from nonresponders to GLPhe as above. No antibody was detected by the ELISA assay in B10 and B10.A congenic inbred strains which by the T cell proliferation assays (Schwartz et al, 1976) and antigen binding assays (Dorf et al, 1975 and Stimpfling and Durham, 1972) were shown to be nonresponders. B10.A(5R) mice are responders to GLPhe therefore production of anti - GLPhe antibody is quite prominent at approximately day 28 to day 49 after immunization as compared to the nonresponders B10 and B10.A (Figure 5). The response of the inbred congenic mice (B10, B10.A, etc.) compared to the inbred mice (Balb/c) is not as prominent due to the recombination events that took place during mating to produce these recombinant strains. Mice of different haplotypes differ in their complementation ability (Dorf and Benacerraf, 1975).

The synthetic polypeptide GLPhe was also conjugated to CGG via a variation on the Schiff base reaction. 100 ug. ip. of this GLPhe - CGG in FCA was administered to the mouse strains of Balb/c, C57Bl/6J, C_3H/Hej, SJL, B10, B10.A, and B10.A(5R). By attaching the small tripeptide to an immunogenic carrier, in this case CGG, allowed not only the responding strains to respond even more but also allowed the nonresponder strains to achieve some degree of response to GLPhe via production of anti - GLPhe antibodies. (Figures 6 & 7)

(III) Induction of Suppression

Since the use of the GLPhe - CGG enabled us to bypass the "gene complementation" requirement to elicit the response,

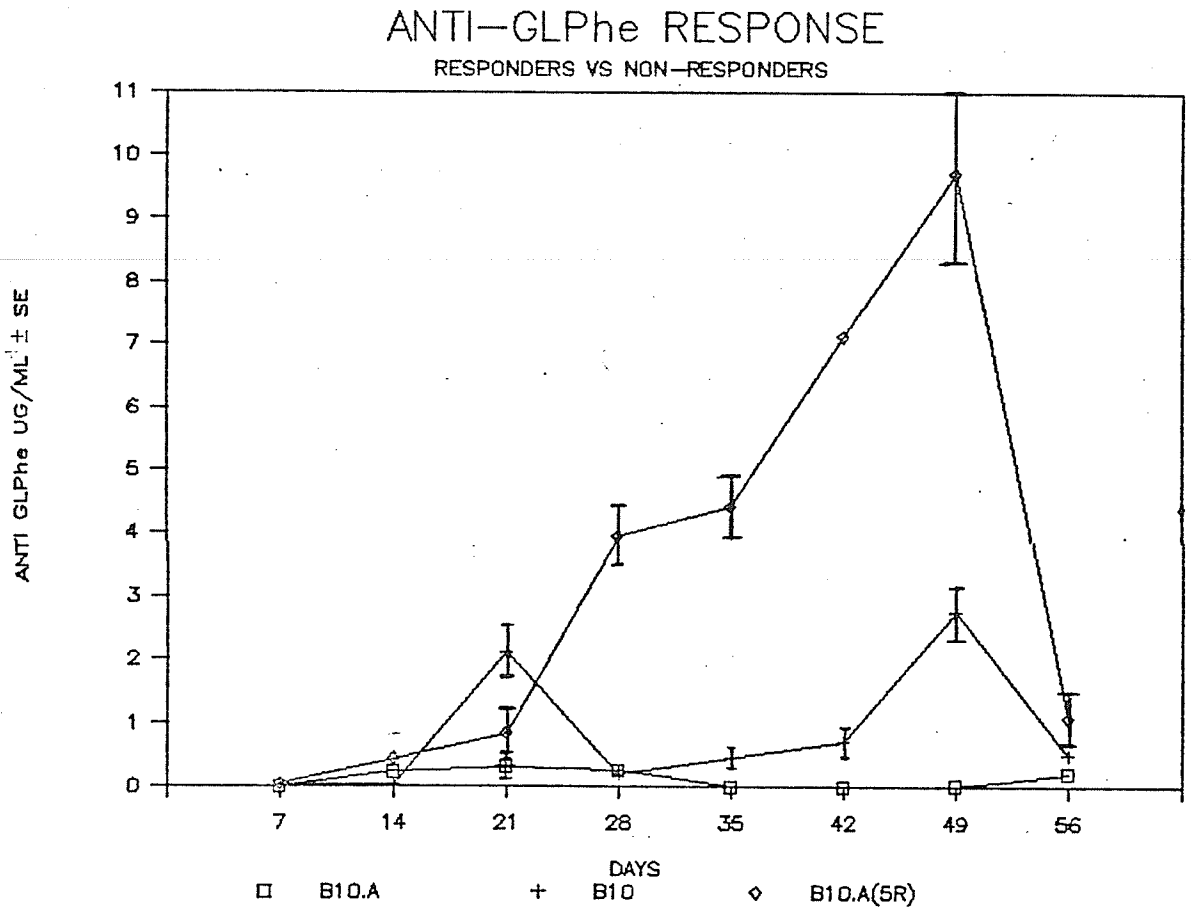
ANTI—GLPhe RESPONSE
RESPONDERS VS NON—RESPONDERS
ANTI GLPhe UG/ML ± SE
11
10
9
8
7
6
5
4
3
2
1
0
7
14
21
28
35
42
49
56
DAYS
B10.A
B10
B10.A(5R)

FIGURE 5

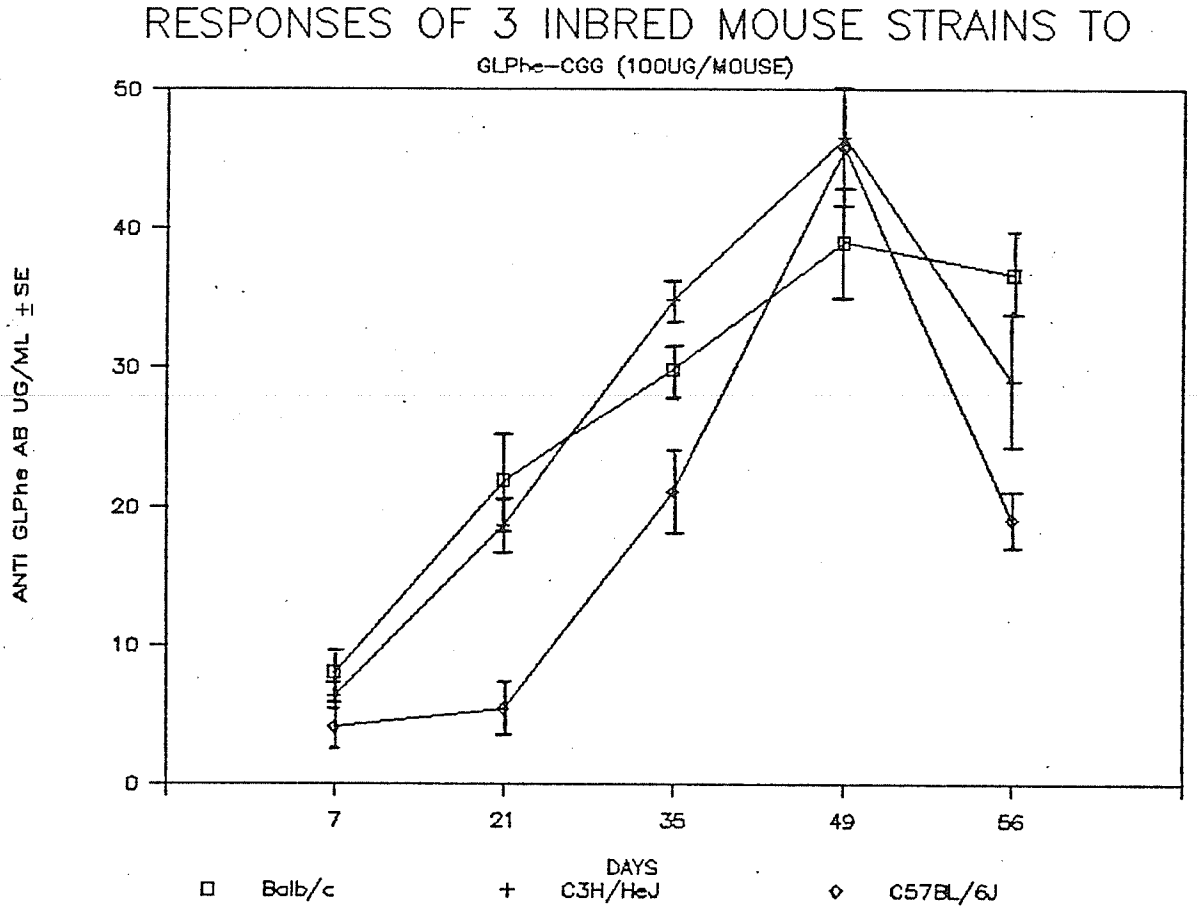
RESPONSES OF 3 INBRED MOUSE STRAINS TO
GLPhe-CGG (100UG/MOUSE)
ANTI GLPhe AB UG/ML ±SE
50
40
30
20
10
0
7
21
35
49
56
DAYS
Balb/c
C3H/HeJ
C57BL/6J

FIGURE 6

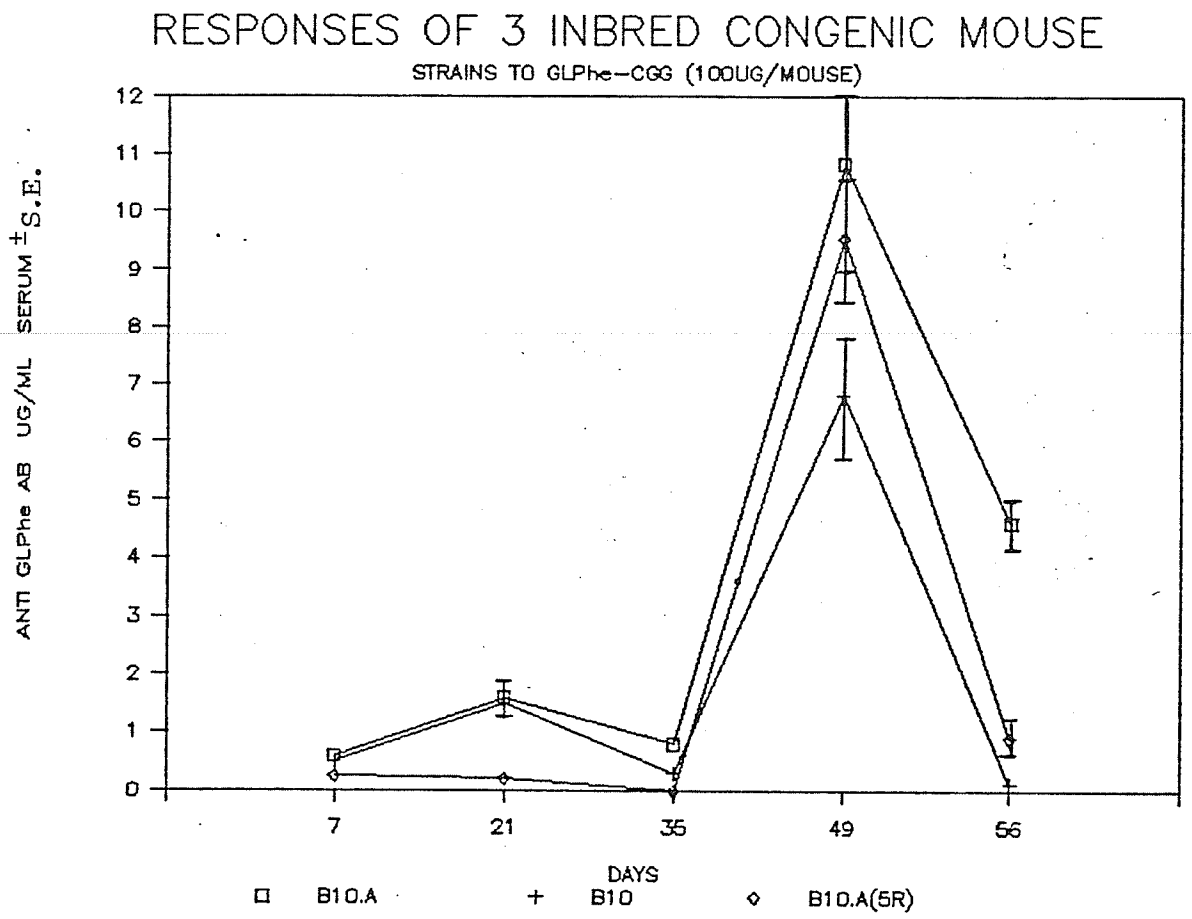
RESPONSES OF 3 INBRED CONGENIC MOUSE
STRAINS TO GLPhe-CGG (100UG/MOUSE)
ANTI GLPhe AB UG/ML SERUM ±S.E.
12
11
10
9
8
7
6
5
4
3
2
1
0
7
21
35
49
56
DAYS
B10.A
B10
B10.A(5R)

FIGURE 7

i.e. production of anti- GLPhe antibodies, this conjugated material provided an excellent starting point to determine if unresponsiveness to the terpolymer GLPhe is due to suppressor cell induction. Since both nonresponders, C57Bl/6J and C_3H/Hej mice, gave a prominent response to GLPhe when immunized with GLPhe - CGG, we have examined the ability if GLPhe to suppress the response if administered before the conjugate. As mentioned above, these two mouse strains, even though they are nonresponders to GLPhe, will respond to GLPhe - CGG.(as seen in Fig. 6)

Pre - immunization with 100 ug. GLPhe (iv) followed 2 weeks later with a challenge of 100 ug. GLPhe - CGG in FCA (i.p.) suppressed the quantity of anti - GLPhe antibody normally produced against the GLPhe - CGG. The ability to suppress the GLPhe - CGG response by administering a pre - immunizing GLPhe (i.v.) injection may be due to the induction of suppressor cells. (Figs.8a & 8b)

Another group of mice, received two doses of 100 ug. of GLPhe (i.v.) two weeks apart and another fourteen days after the second GLPhe dose they received an injection of GLPhe - CGG. As shown in Figure 8a and 8b, the second dose of GLPhe in the preimmunization schedule increased the suppression of the anti - GLPhe response. This increased suppression appears to be more prominent in the C_3H/HeJ than in the C57Bl/6J strain.

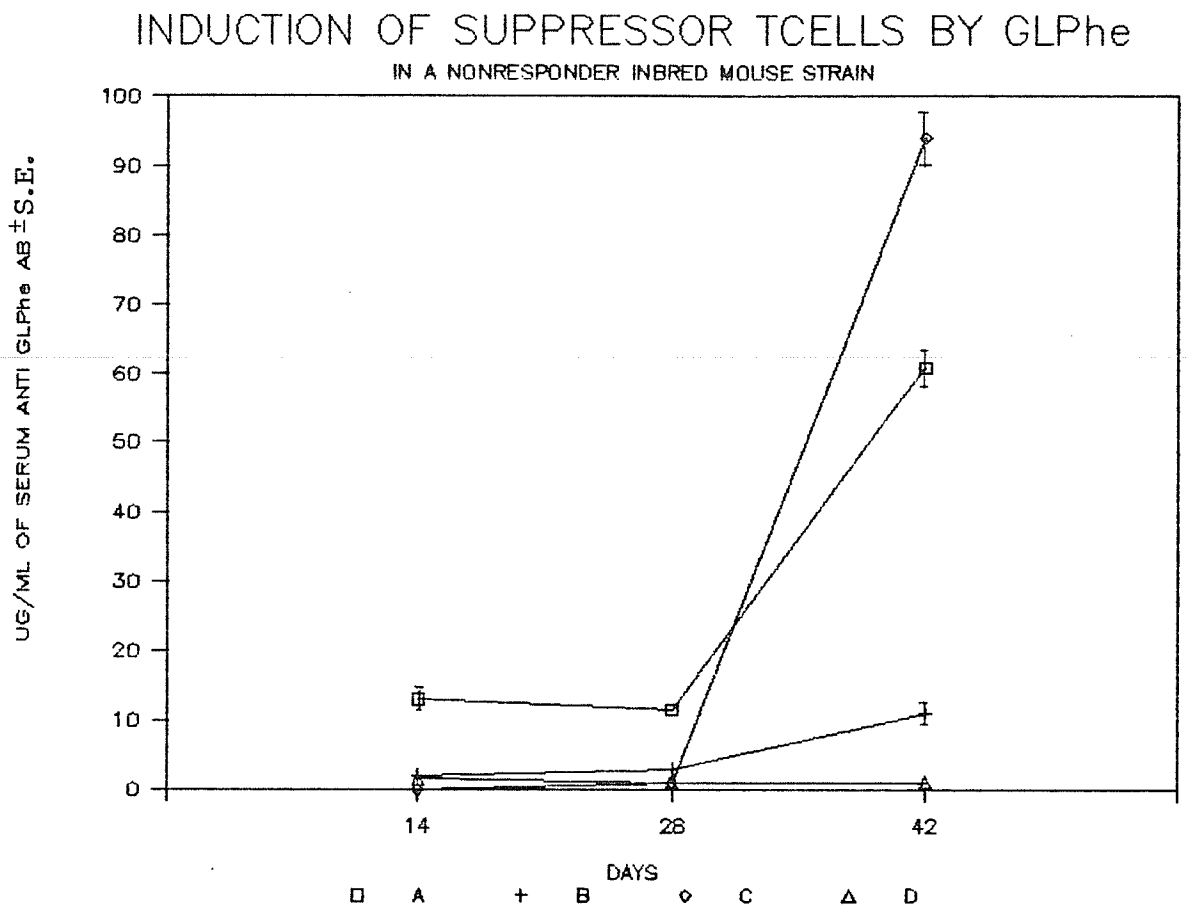

FIGURE 8a

LEGEND: A = GLPhe - CGG

B = GLPhe $\xrightarrow{14d}$ GLPhe - CGG

C = cyclophosphamide $\xrightarrow{2d}$ GLPhe $\xrightarrow{14d}$ GLPhe - CGG

D = GLPhe $\xrightarrow{14d}$ GLPhe $\xrightarrow{14d}$ GLPhe - CGG

Mouse Strain = C57 Bl/6J

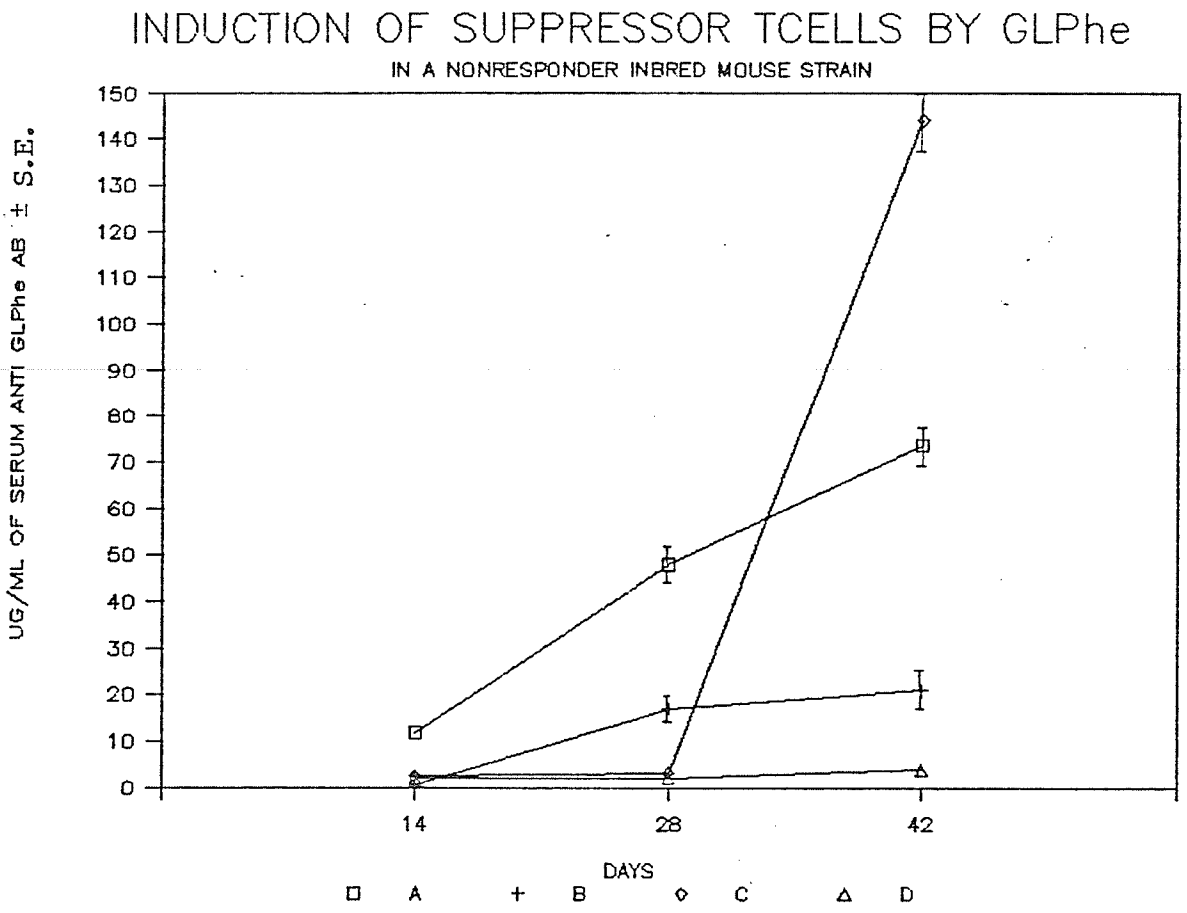

FIGURE 8b

LEGEND: A = GLPhe - CGG

B = GLPhe $\xrightarrow{14\ d}$ GLPhe - CGG

C = cyclophosphamide $\xrightarrow{2\ d}$ GLPhe $\xrightarrow{14d}$ GLPhe - CGG

D = GLPhe $\xrightarrow{14d}$ GLPhe $\xrightarrow{14d}$ GLPhe - CGG

Mouse strain = C3H/HeJ

(IV) Evidence for Suppressor Cell Involvement in Nonresponder Strains to GLPhe

The administration of cyclophosphamide in low doses provided further evidence for T suppressor cell induction. Cyclophosphamide was administered at a dose of 2.0 mg./kg. mouse i.p. followed two days later by an injection of 100 ug. GLPhe (i.v.). The same group was challenged with 100 ug. of GLPhe - CGG/FCA emulsion intraperitoneally, two weeks later. As shown in Figures 8a and 8b, the administration of low dose cyclophosphamide blocked the suppressive effect exerted by the GLPhe preimmunization and, in addition, displayed an increased enhancing effect on the anti- GLPhe antibody response.

Cyclophosphamide when given two days before 100 ug. GLPhe (i.v.) also displayed an enhancing effect on the anti - GLPhe antibody response as compared to the response when 100 ug. GLPhe (i.v.) was administered alone (Fig. 9a & 9b). Since cyclophosphamide effectively eliminates the T suppressor cell population, this is additional evidence that unresponsiveness in certain strains of mice is due to the induction of T suppressor cells directed towards the terpolymer GLPhe.

(V) Specificity of Suppression

The specificity of suppression of the GLPhe (i.v.) dose given as the preimmunization treatment was also traced. Fourteen days after the pre - immunization step of 100 ug. GLPhe (i.v.), a BSA injection

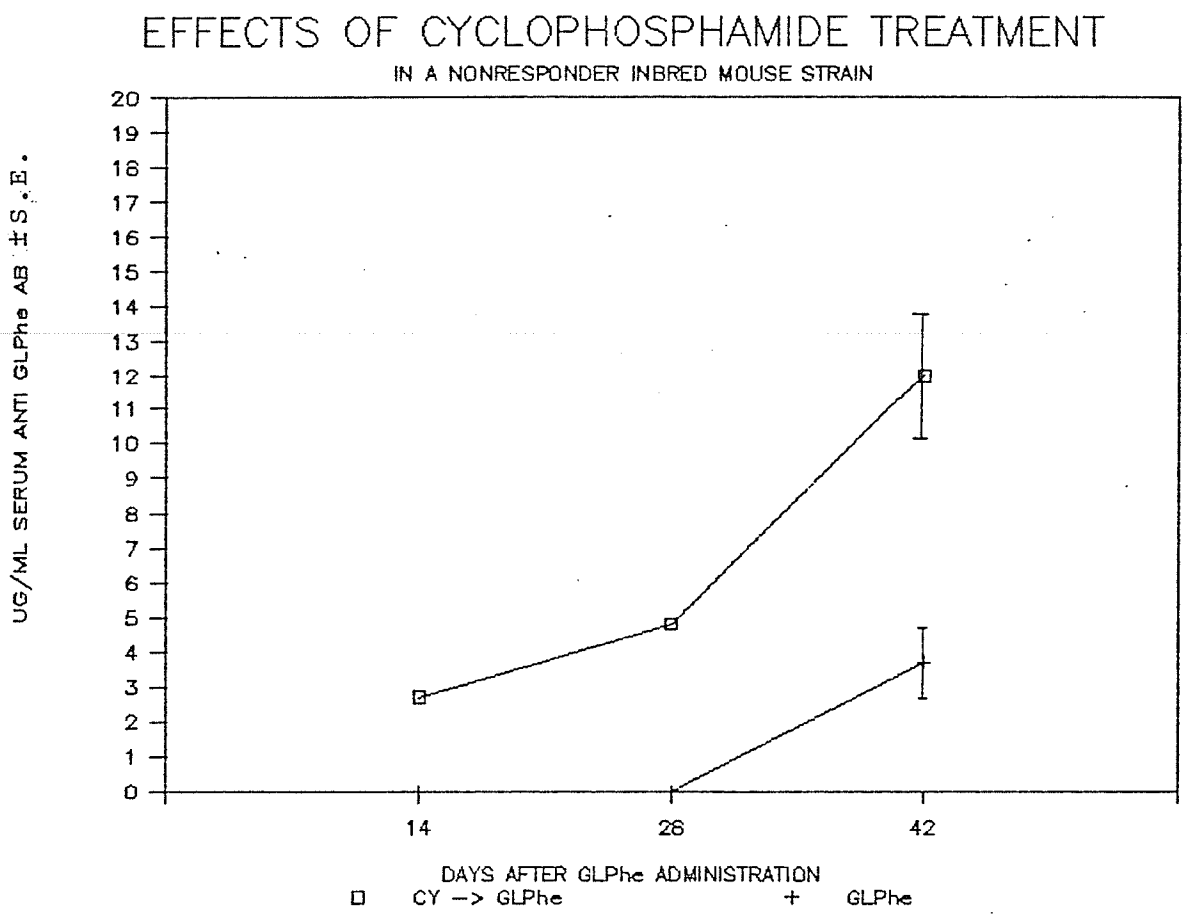

FIGURE 9a

Mouse strain ; C3H/HeJ

* When no bars for S.E. appear, the S.E. was too small to be pictated graphically.

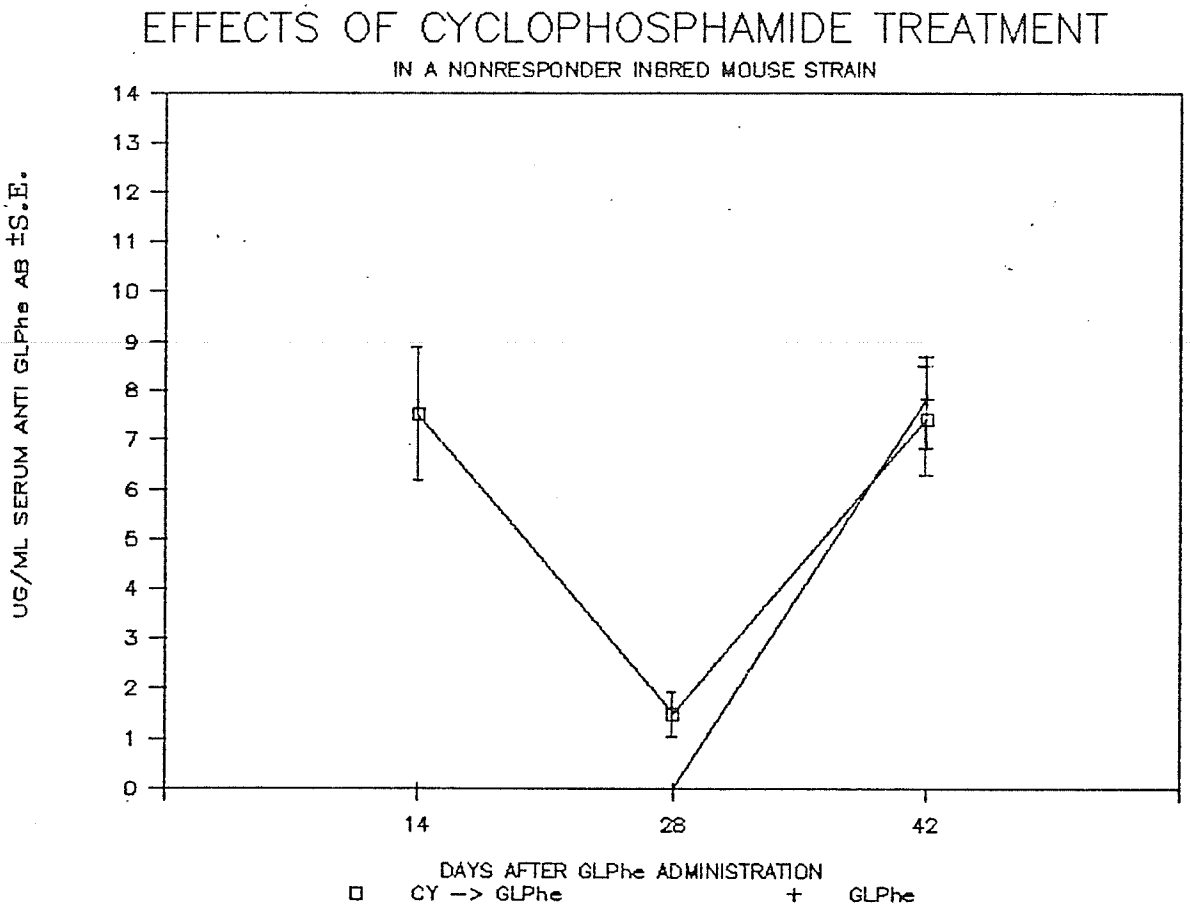

FIGURE 9b

Mouse strain: C57B1/6J

of 100 ug.(i.p.) was administered. The control used was one group of mice which received only 100 ug BSA (i.p.) This serum was tested for both anti - GLPhe antibody and anti - BSA antibody responses.

The anti - BSA antibody response after a pre - injection of GLPhe was found to be equal to the control anti- BSA antibody response. (Fig. 10a & b) Therefore, the preimmunization treatment of 100 ug. GLPhe (i.v.) was specific in its suppression i.e.it only effected anti - GLPhe antibody responses, not antibody responses to other foreign antigens.

(VI) Six - hour Complex Production and Uptake

Six hour serum was produced in the strains Balb/c, C57Bl/6J, and C_3H/Hej by injection of 100 ug. GLPhe in FCA intraperitoneally. These mice were bled 6 hours later by the cardiac puncture method. This serum was tested in the reverse immune cytoadherence assay (RICA) developed by Paraskevas et al. As shown in Table 1, all the above four inbred mouse strains produced their own 6 hour complexes and these complexes were taken up by the appropriate syngeneic spleen cells with the exception of C57Bl/6J and SJL strains. The C57Bl/6J uptake of its own complexes was borderline. It was also found that T cells from C57Bl/6J were not capable of uptake of complexes from both Balb/c and C_3H/HeJ mice. However, the C57Bl/6J "6 hour complexes" had the same level of uptake by T cells isolated from Balb/c and C_3H/HeJ spleen cells as these strains had with their

TABLE 1

INDUCTION OF "6 HOUR COMPLEXES"

TO GLPhe IN RESPONDER AND

NON-RESPONDER MICE

	SPLEEN CELLS			
SERUM	Balb/c	C57Bl/6J	C3H/HeJ	SJL
Balb/c	87	21	75	18
C57Bl/6J	79	42	71	0
C3H/HeJ	92	0	74	22
SJL	86	16	89	5

Legend: Assay for presence of "6Hour complexes"

The serum of mice collected 6 hours after immunization (6HS) contains complexes of Ig and antigen ("6 hour complexes"), which are strongly cytophilic for T cells. The T cells normally carry no easily detectable surface Ig but after the uptake of the complexes they become strongly Ig^+. When spleen cells are incubated with 6HS, a net increase of Ig^+ cells is detectable due to the uptake of the "6 hour complexes" by T cells. This increase amounts to 80-90 Ig^+ cells per 1000 spleen cells. Since this assay is highly sensitive and extremely reproducible, this detection method is very reliable. The technique has been used for over 10 years.

* Numbers represent increases of Ig^+ cells/1000 spleen cells.

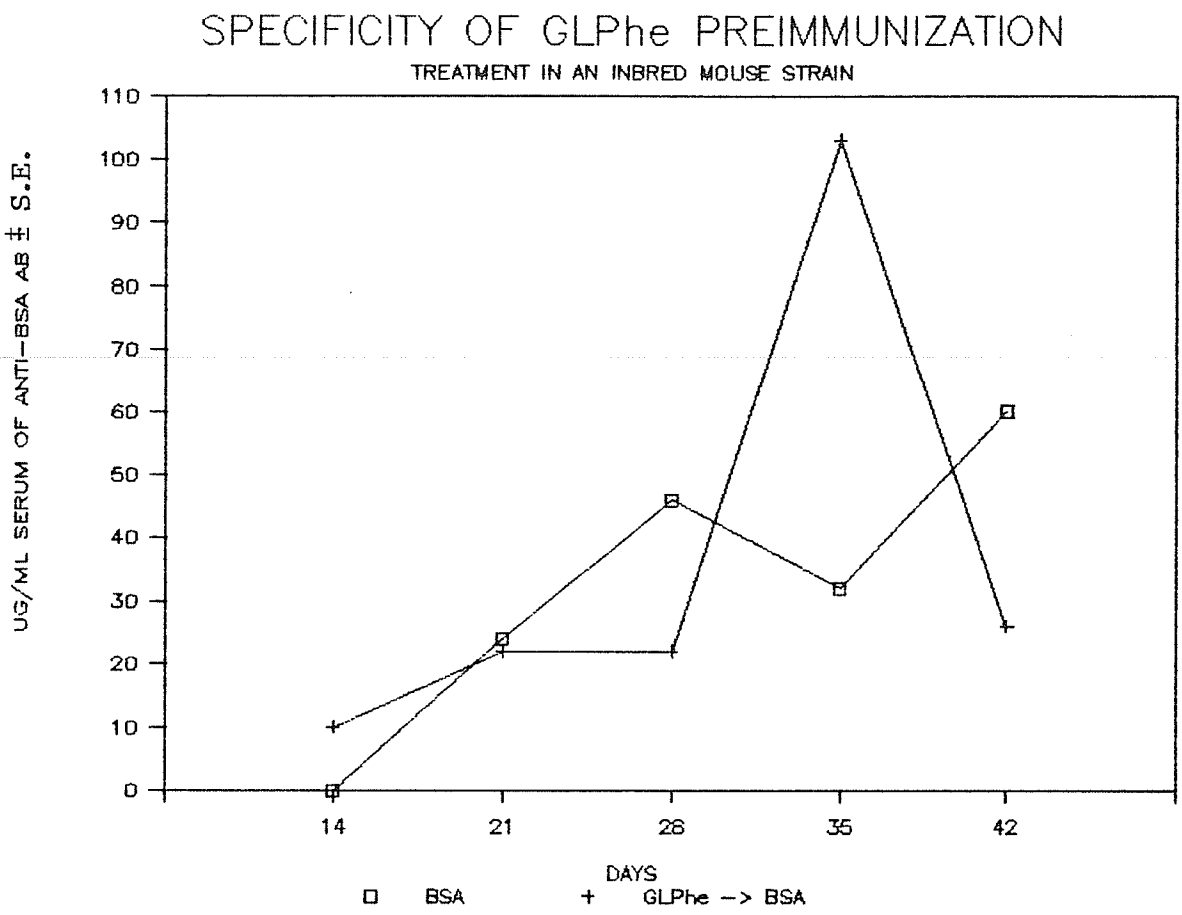

FIGURE 10a

Mouse strain : C57B1/6J

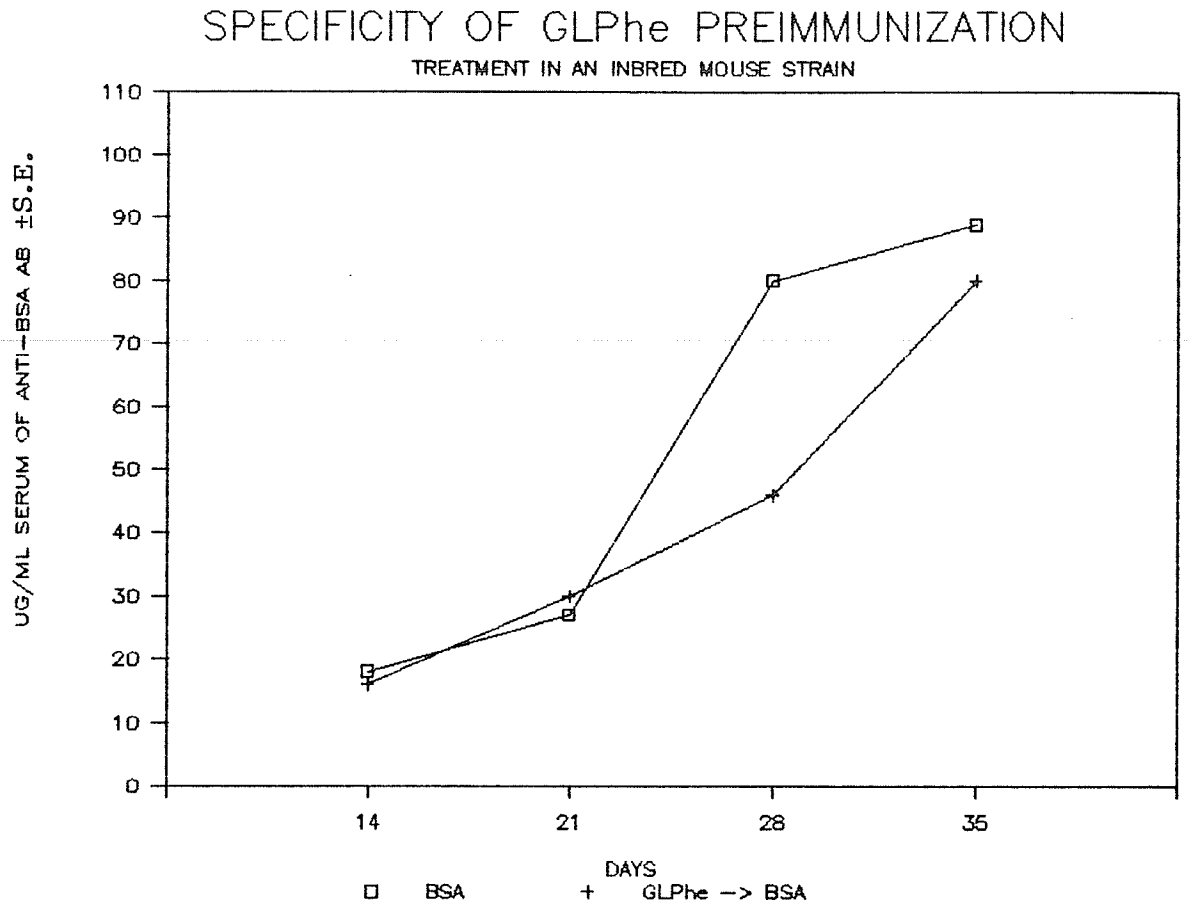

FIGURE 10b

Mouse strain : C3H/HeJ

respective syngeneic T cells.

These data demonstrate that both responders (Balb/c) and non-responders (C_3H/HeJ and C57Bl/6J) can produce "6 hour complexes" following immunization with GLPhe in FCA. However, the two non-responder strains can be distinguished in the capacity of their T cells to take up the complexes. While the C_3H/HeJ T cells takes up complexes from the other two strains, the C57Bl/6J are defective in this respect.

(VII) Conversion of Nonresponder to Responder via 6 Hour Serum Transfer

The transfer of 6 hour serum between responder and nonresponder strains was performed by developing 6 hour complexes to GLPhe in various inbred strains and transferring 0.3 ml. of this serum into the appropriate mouse strains. Two hours later, a subimmunogenic dose of 50 ug. GLPhe, was administered intraveneously and the animals were bled every 7 days from day 0 to day 42.

The first transfer of 6HS performed is shown in Figure 11. Balb/c 6HS was transferred into a syngeneic recipient and an enhancement of anti - GLPhe antibodies was exhibited. (Fig. 11) This enhancement was compared to syngeneic recipients which received only 50 ug. GLPhe (i.v.) and to syngeneic recipients who received a transfer of syngeneic NMS. (Fig. 11)

6 hour serum from a GLPhe responder strain, i.e.Balb/c, converted the nonresponder strain C_3H/Hej to a responder as measured by its

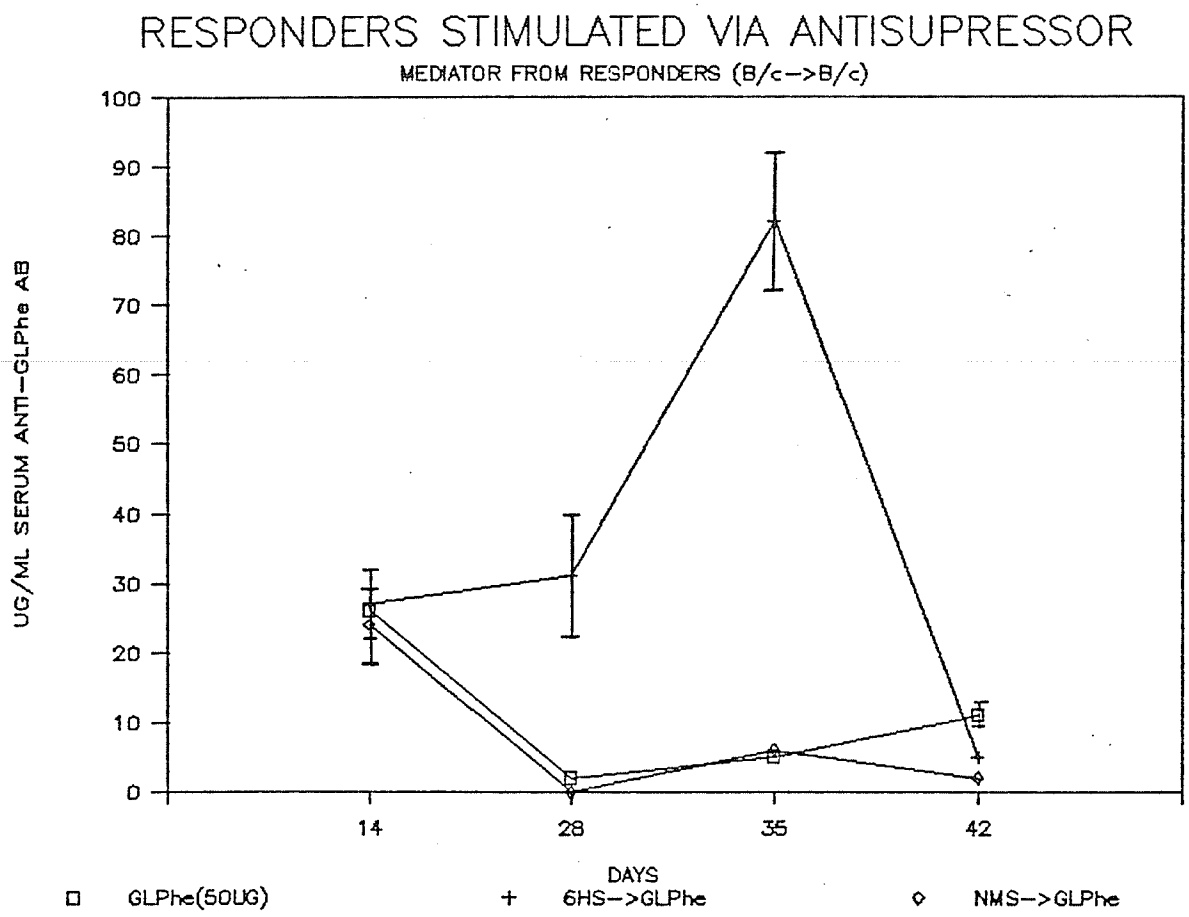
RESPONDERS STIMULATED VIA ANTISUPRESSOR
MEDIATOR FROM RESPONDERS (B/c->B/c)
UG/ML SERUM ANTI-GLPhe AB
100
90
80
70
60
50
40
30
20
10
0
14
28
35
42
DAYS
GLPhe(50UG)
6HS->GLPhe
NMS->GLPhe

FIGURE 11

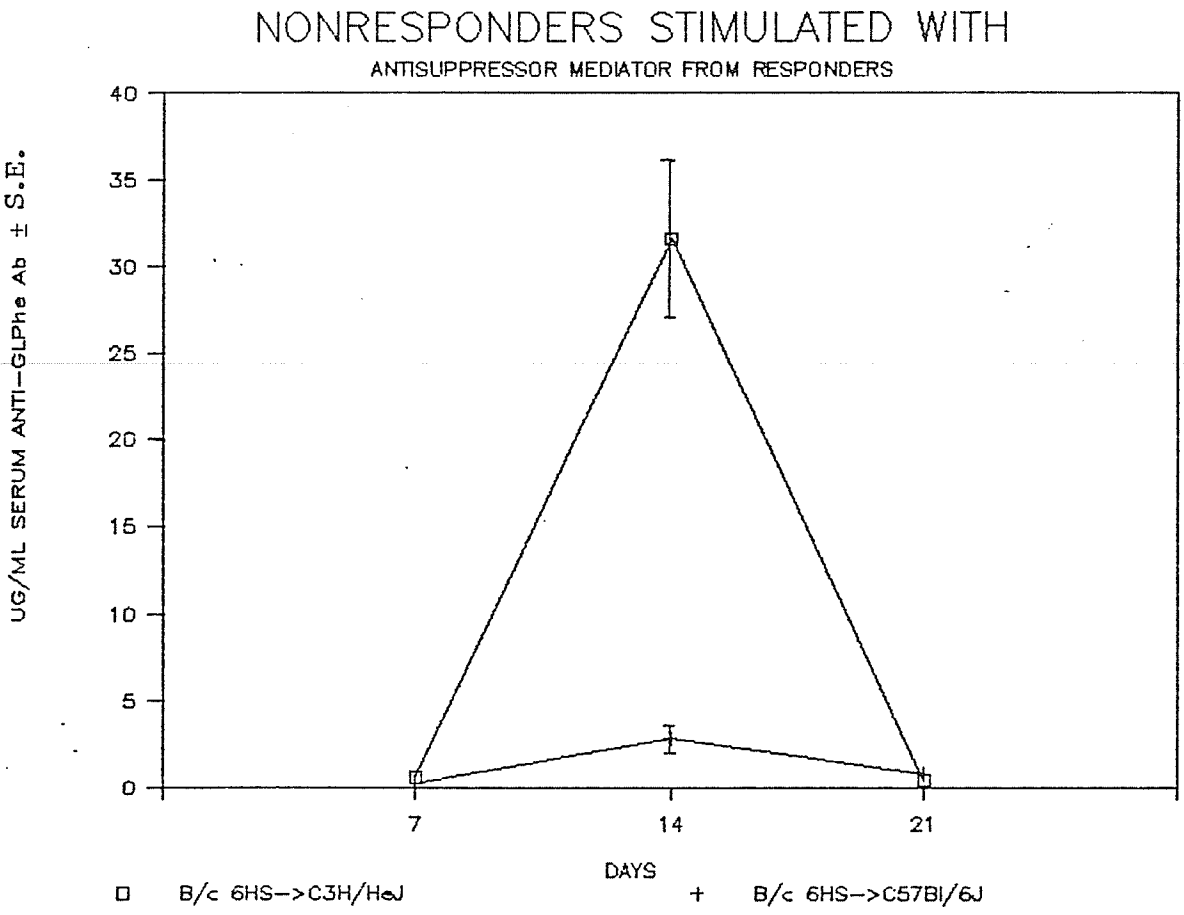
NONRESPONDERS STIMULATED WITH
ANTISUPPRESSOR MEDIATOR FROM RESPONDERS
UG/ML SERUM ANTI-GLPhe Ab ± S.E.
40
35
30
25
20
15
10
5
0
7
14
21
DAYS
□ B/c 6HS->C3H/HeJ
+ B/c 6HS->C57Bl/6J

FIGURE 12

production of anti - GLPhe antibodies (Fig. 12). Although this finding is not unexpected since by the RICA data (Table 1) C_3H/HeJ were shown to uptake complexes of syngeneic as well as allogeneic origin, the uptake of complexes by T cells may not always mean enhancement or activation, since even though the C_3H/HeJ mouse can take up it's own complexes it is still a nonresponder to GLPhe. Only a responders' complex will work to convert a nonresponder into a responder.

The transfer of Balb/c 6 hour serum into C57Bl/6J mice did not convert these nonresponders into responders.(Fig. 12) This was the expected result since C57Bl/6J showed no uptake of complexes by the RICA method (Table 1) and therefore no anti - GLPhe antibody response would be expected.

Six hour sera from the nonresponders C57Bl/6J and C_3H/HeJ were also transferred into nonresponders C_3H/HeJ and C57Bl/6J respectively. As shown in Figure 13a, the transfer of C_3H/HeJ six hour serum into a C57Bl/6J recipient converted this nonresponder strain into a responder. The C57Bl/6J six hour serum however transferred into the nonresponder C_3H/HeJ, did not produce a significant anti - GLPhe response (Fig. 13b).

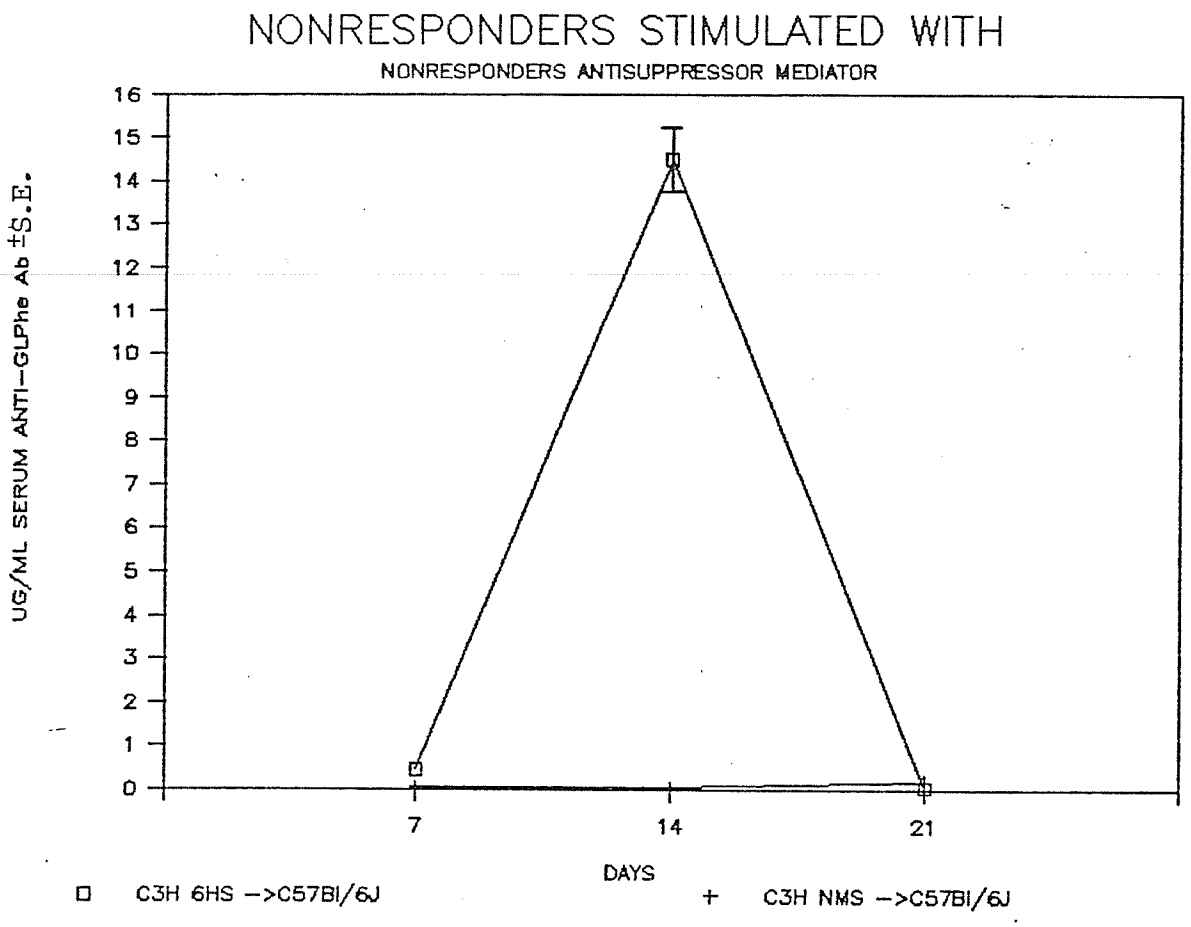
NONRESPONDERS STIMULATED WITH
NONRESPONDERS ANTISUPPRESSOR MEDIATOR
UG/ML SERUM ANTI-GLPhe Ab ±S.E.
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
7
14
21
DAYS
C3H 6HS ->C57Bl/6J
C3H NMS ->C57Bl/6J

FIGURE 13a

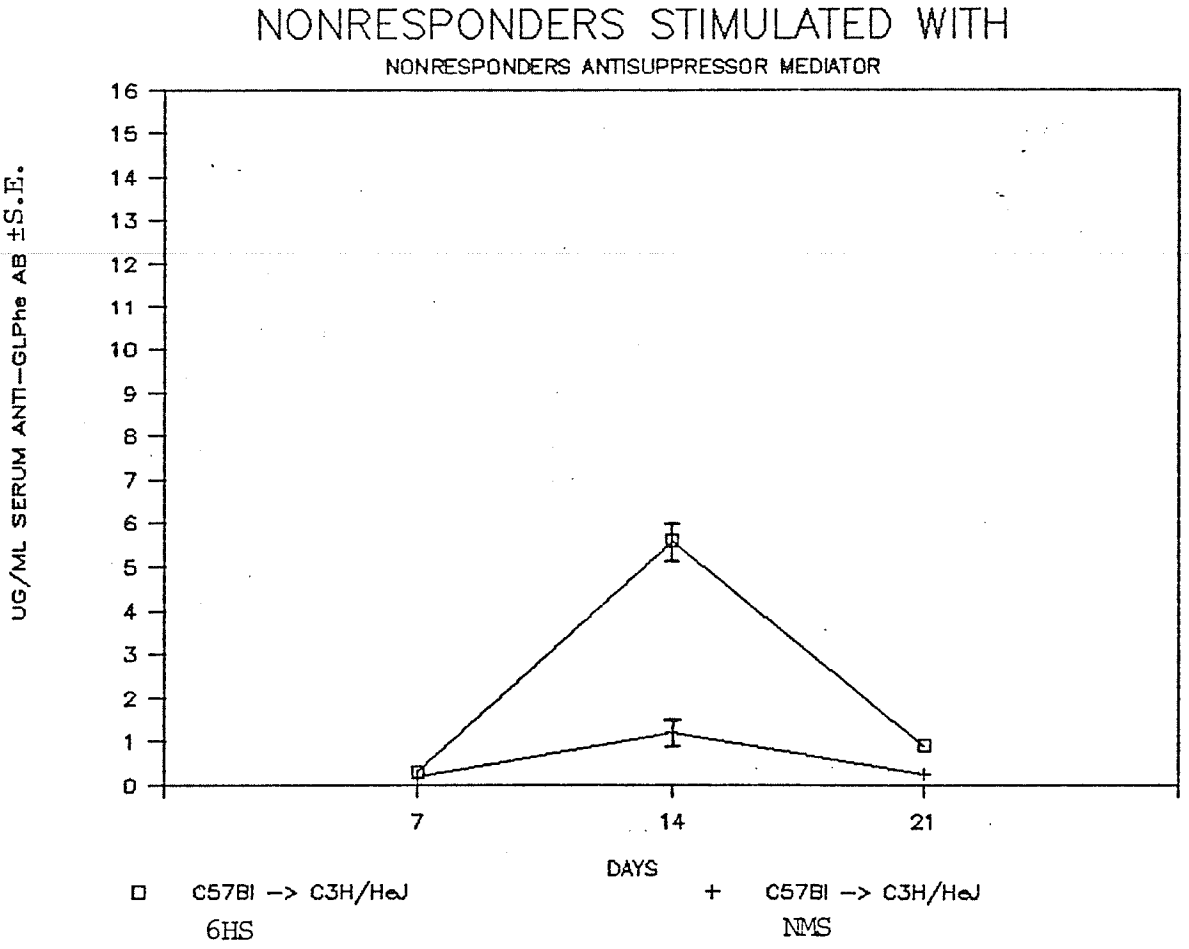
NONRESPONDERS STIMULATED WITH
NONRESPONDERS ANTISUPPRESSOR MEDIATOR
UG/ML SERUM ANTI-GLPhe AB ±S.E.
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
7
14
21
DAYS
□ C57Bl -> C3H/HeJ
6HS
+ C57Bl -> C3H/HeJ
NMS

FIGURE 13b

DISCUSSION

Until 1972, it was generally assumed that a single histocompatibility - linked Ir gene was required for responsiveness to an antigen. It was in 1972 that Stimpfling and Durham discovered that the immune response of their mice to the alloantigen H - 2.2 was controlled by two interacting genes localized in the histocompatibility complex. An example of dual H - linked gene control of the response to a single antigen was documented by Dorf, Stimpfling, and Benacerraf in 1975 and was termed gene complementation. The only assay systems that were available for evaluation of the response to GLPhe was the antigen binding assay (Dorf, Stimpfling, and Benacerraf, 1975) used in the late 1970's and recently the T cell proliferative assay. (Schwartz et al, 1976) The T cell proliferative assay proves to be a rather cumbersome and complex assay requiring in vivo immunizations and in vitro culture techniques whereas the development of the modification of the ELISA technique utilized in this report is a relatively simple and easy assay to perform when dealing with responsiveness to antigens. By improving the original technique, the construction of a standard curve allowed the conversion of O.D. measurements to a quantitative measure of anti - GLPhe antibody expressed as ug./ml. (Fig. 1).

The responsiveness of the inbred and inbred congenic strains tested were found to be in agreement with the results using these same strains with GLPhe as the antigen in T cell proliferation assays (Schwartz et al, 1976) and by antigen binding assays (Dorf et al,

1975) (Stimpfling and Durham, 1972).

Mice that lack one of the complementing genes, alpha or beta, are considered nonresponders to GLPhe. By conjugating GLPhe to CGG (Cheung et al, 1977) the same mouse strains as above were tested for their responsiveness to GLPhe after administration of the GLPhe - CGG conjugate. By attaching GLPhe, to an immunogenic carrier, in this case CGG, the responder strains responded to a greater degree than with only an immunization with GLPhe. As well, the nonresponder strains to GLPhe achieved a degree of responsiveness via the production of anti - GLPhe antibodies (Fig. 6 & 7). This demonstrates that by utilizing an immunogenic carrier, the antigen specific T_H cell may be bypassed therefore allowing a nonresponder animal to now respond to the GLPhe. Results such as this have also been demonstrated in the GAT system.

Serological evaluation of responders and nonresponders to GAT that were immunized with the conjugate GAT - MBSA demonstrate that, in vivo, MBSA acts as an immunogenic carrier just as CGG does in the GLPhe system. (Durham, 1972) In otherwords, responders that are immunized with GAT - MBSA produce elevated levels of IgG GAT - specific plaques. In addition, nonresponders produced IgG GAT - specific plaques in response to a GAT - MBSA immunization.
The same result occurred in vitro with the addition of GAT - MBSA into spleen cell cultures. (Kapp et al, 1973)

Although unresponsiveness to GAT has been shown to be due to T_S induction, conclusive evidence that the same is true for GLPhe unresponsiveness has never been obtained. We have therefore

investigated first whether the unresponsiveness to GLPhe in C_3H/HeJ and C57Bl/6J is due to T_S. We have used two approaches:

(a) suppression of the response observed after GLPhe - CGG immunization, by pre - immunization with GLPhe

(b) by cyclophosphamide.

Utilizing the first approach, the data demonstrated that pre-immunization suppressed the anti - GLPhe antibody response to a subsequent injection of GLPhe - CGG. (Fig.6) In otherwords, induction of suppression occurred. A similar protocol which obtained much the same results was used in Benacerraf's lab with GAT and GT. (Debre,P. et al, 1975, 1975, 1976)

In the GAT system it was found that pre - injection of soluble GAT specifically suppressed the ability of nonresponder mice to develop an anti - GAT response to a subsequent challenge with GAT - MBSA in vivo. (Kapp et al, 1974) It was also determined that spleen cells from GAT - primed mice specifically suppressed in vitro spleen cell cultures of responders incubated with GAT - MBSA. This suppression was shown to be caused by specific Ly 2^+ T_S cells. (Kapp et al, 1974)

This same phenomena was demonstrated in the GT system. Certain mouse strains which are nonresponders to GT can produce an anti - GT response in response to the administration of GT- MBSA. A pre - immunization of 100 ug. of GT specifically decreased the ability of mice to produce an anti - GT PFC response to a challenge injection of GT - MBSA. (Debre, P. et al, 1975) Upon further examination of suppressor cell induction in the GT system, it was noted that the GT

pre - immunization protocol inhibited the GT - MBSA response only in certain mouse strains. Strains bearing H -2^k, H-2^d, or H-2^f haplotypes but not H-2^b or H-2^s haplotypes were specifically suppressed by GT - preimmunization regardless of the genes contributed by the B10 background in the congenic resistant strains used. (Debre,P. et al. 1975) This group concluded that immune suppression could not account for nonresponsiveness in all cases although nonresponsiveness in the GT system depends on the capacity to develop GT - specific suppression which is inherited as a dominant trait. The genes controlling this trait are GT - specific Is genes which have been mapped in the I region of the H-2 complex between the K and D regions. (Debre, P.,1976) As noted in the introduction, the gene complementation in the two different systems, GLPhe and GT, although appears to be comparable are actually completely opposite in their responsiveness/nonresponsiveness patterns. In the case of response to GLPhe, complementation was demonstrable by the responsiveness of recombinant strains derived from nonresponder strains while in the GT system, recombinants between suppressor mice lost their ability to generate suppressor cells but still could not be demonstrated to behave as unequivocal responders. However, induction of suppressor cells as shown in Fig. 8 in the GLPhe system and above in the GAT and GT system does occur. (Benacerraf and Dorf, 1976)

In this report, the specificity of suppression was also examined to determine whether the GLPhe pre - immunization suppressed not only responses to GLPhe - CGG but responses to other antigens. The same protocol as suppression induction was utilized. Induction of

suppression in the GLPhe system was found to be specific for GLPhe and did not affect the response to other antigens such as BSA. (Fig. 10)

To provide further evidence that unresponsiveness to GLPhe was due to T suppressor cell induction, the protocol of administration of low dose cyclophosphamide to non - responder mice before soluble antigen immunization was utilized. The injection of low doses of cyclophosphamide was first found to preferentially inhibit suppressor cell activity in Jones - Mote DTH reactions and contact sensitivity. (Polak et al, 1974) Debre et al (1976) used this cyclophosphamide protocol in the GT system and found that there was no suppressive effect after GT/cyclophosphamide administration to a subsequent injection of GT in the form of GT - MBSA. As well, the cyclophosphamide treatment even allowed the nonresponder to develop a primary immune response to a GT challenge alone. These cyclophosphamide induced responses were however, not characterized by the high levels of antibody detected in genetic responder animals. Our data with the GLPhe system showed results similar to the ones described here; low dose cyclophosphamide blocked the suppressive effect exerted by the GLPhe pre - immunization and displayed an increased enhancing effect on the anti - GLPhe antibody response after a challenge with GLPhe - CGG. (Fig. 8) Administration of cyclophosphamide to nonresponders before a soluble GLPhe dose also allowed a primary immune response to occur to GLPhe. (Fig. 9) This immune response was apparent in the C_3H/HeJ strain throughout the 42 days post-immunization period although in the C57Bl/6J, this cyclophosphamide treatment seemed to be effective only until day 14

post - immunization. This data can be explained as in Debre's GT system. Debre showed that the cyclophosphamide induced responses do not produce the same high levels of antibody as the genetic responder animals. (Debre et al, 1976) This is due to the cyclophosphamide having a limited time effect only on the particular suppressor cell population present at the time of administration of cyclophosphamide.

Another reason for the lowered antibody levels is animals that received cyclophosphamide are given a subimmunogenic dose intraveneously without FCA whereas responder animals are determined by an intraperitoneal injection of GLPhe in FCA. The degree of "suppression relief" also varies in mice of different haplotypes.

Since cyclophosphamide effectively eliminates the T suppressor cell population, this will be the first time that T_S cells have been shown to be induced in GLPhe non - responders and therefore, in contrast to GAT and GT, Is genes have never before been implicated in nonresponsiveness to GLPhe. Since two Ir genes are needed for anti - GLPhe responses, nonresponsiveness have been considered to be due to the lack of one of the two genes to this date.

Another interpretation of nonresponsiveness which deals with the lack of one of the two genes, i.e. no gene complementation, could be defined within the antisuppression pathway described by Paraskevas et al (1984). The "proper" induction of the antisuppressor pathway will lead to immunity or responsiveness in the GLPhe system. Any interference with this pathway will result in unresponsiveness. In this report we have cited defects in the antisuppressor pathway of nonresponders to GLPhe. By assaying inbred mouse strains by the RICA

techinique (1971), it was shown that all strains tested, responders and nonresponders alike, produced "6 hour complexes" to GLPhe. However, RICA tests for the presence of "6 hour complexes" it does not determine whether these complexes are functional. The difference between the strains, according to the RICA data, occurred at the level of T cell uptake. (Table 1) It has been demonstrated before that "6 hour complex" uptake by T cells is not H-2 restricted and therefore these defects in GLPhe nonresponders are real defects in their antisuppression mechanism. The most notable defects were in the two nonresponders, C57Bl/6J and C_3H/Hej. The C57Bl/6J defect was due to failure of their T cells to uptake both syngeneic and allogeneic "6 hour complexes". The C_3H/Hej T cells on the other hand could take up syngeneic and allogeneic complexes but could not activate their T cells to continue in the antisuppressor pathway once the complexes were bound. This evidence demonstrates that unresponsiveness in the GLPhe system, in this case, may be due to interference in the function of the antisuppressor pathway and this type of defect may apply to unresponsiveness in other antigen systems as well. Since there are two stages in the antisuppressor pathway i.e. induction, which is formation of the complex, and the effector stage, which is activation of the T cell that takes up complexes, defects in any of these stages will result in T_S cell induction and unresponsiveness.

The last experiments performed were transferring "6 hour complexes" into inbred strains. These 6 hour serum experiments were an attempt to determine whether the complexes or the T cell taking up the complexes are defective in the nonresponders. In

otherwords, the following experiments were performed in order to test the hypothesis of gene complementation according to antisuppression induction and effector stages. For this testing, the assay of enhancement of antibody production via 6 hour serum using subimmunogenic stimulations was utilized.

When the responder Balb/c 6 hour serum was transferred into the nonresponder C57Bl/6J, no responsiveness occurred as expected since evidence by RICA showed that the C57Bl/6J did not uptake the complexes. Since the induction of complexes is controlled by the I-A molecule and uptake is controlled via the I-E molecule, the C57Bl/6J, which are of haplotype b, do not carry a functional I-E molecule therefore complex uptake does not occur with a responders' functional complex. However, when "6 hour complexes" from Balb/c were transferred into the nonresponder C_3H/Hej, this nonresponder was converted into a responder. (Fig. 12) When Balb/c 6 hour serum is transferred into C_3H/HeJ or C57Bl/6J, the functional capacity of the complexes from Balb/c (induction) and the functional capacity of the T cells taking up the complexes from the C_3H/HeJ or C57Bl/6J (effector stage) are tested.

To demonstrate that gene complementation between two nonresponders in the GLPhe system can produce an active responder to GLPhe, 6 hour sera from the nonresponders C57Bl/6J and C_3H/HeJ were transferred into each other and assayed for anti - GLPhe antibody responses. As shown in Figure 13, when C_3H/HeJ 6 hour serum was transferred into the C57Bl/6J recipient, an elevated anti - GLPhe antibody response occured as compared to the transfer of C_3H/HeJ NMS

into C57Bl/6J. The RICA data showed no uptake by the C57Bl/6J T cells of the C_3H/HeJ 6 hour complexes although this may be a matter of sensitivity of the method. In otherwords, some uptake may take place although undetectable by RICA. The ELISA method used in these experiments is very sensitive and detects the consequence of the uptake i.e. antibody. This demonstrates that in addition to the RICA data, the C_3H/HeJ "6 hour complex" is functional. Although it would not be expected that the C57Bl/6J could properly accept this functional complex since it is void of a functional E molecule, this did occur.

One explanation for this data is that in mice that do not express the E molecule, the A molecule takes over complementation regulation. In this case, the complementation is between the A^k of the C_3H/HeJ making the complex and the A^b on the C57Bl/6J taking up the complex. Therefore, gene complementation may not be simply between the I-A and I-E subregions as much as between the components of the Ia molecule i.e. between the alpha and beta chains of the A molecule ($A \rightarrow A_B$) or E molecule ($E \rightarrow E_B$). The "typical" or "classical" complementation is between I-A (E_B) for induction of anti-suppression and I-E (E_α) for the effector stage of antisuppression or complex uptake while the I-A (A_α) and I-A (A_B) complementation takes place when the E molecule is not expressed.

In other cases, hybrid complementations may take place like the above in the C_3H/HeJ 6 hour serum into the C57Bl/6J recipient for uptake. Here the complementation is $A_\beta{}^k \rightarrow A^b$ or $A^k \rightarrow A_\beta{}^b$. Therefore, the haplotypes of the molecules involved in complementation

may be important. In otherwords, certain combinations may produce a greater response than the reverse combination or may not produce a response at all to GLPhe.

Another explanation for the fact that when transferring non-responder "6 hour complexes" into a nonresponder recipient the above result occurs is that a second signal or factor may be required and in the above case supplied. Further experimentation would lead to the answer of this question however it could be postulated that this additional factor could be IL-1 which is produced by macrophages and is important for complex formation. It has been shown that IL-1 also stimulates T cells.

An additional explanation for the above result could be that since the I-A subregion is responsible for complex formation, the uptake of the Balb/c responder complex ($I\text{-}A^d$) may not fit to the C57Bl/6J effector T cell whereas the C_3H/HeJ nonresponder complex ($I\text{-}A^k$) does. (Figs. 12 & 13)

When the nonresponder C57Bl/6J 6 hour serum was transferred into the nonresponder C_3H/HeJ, no enhancement of anti - GLPhe antibody was noted as compared to the transfer of C57Bl/6J NMS into C_3H/HeJ. This is explained by the fact that although the C_3H/HeJ contains functional effector cells, the C57Bl/6J complex may not be functional or may not "fit" to the C_3H/HeJ effector cell or that since this is a case of hybrid complementation, the C57Bl/6J 6 hour serum $\rightarrow$ C_3H/HeJ combination does not produce an anti - GLPhe antibody response.

Although further experimentation utilizing congenic inbred strains would provide additional insight into the explanation of gene

complementation on the basis of the two stages of the antisuppressor pathway, this report gives strong support that antisuppression plays an active role in responsiveness/unresponsiveness. The development of the highly sensitive quantitative technique for detection of GLPhe response (ELISA) will help to unravel the genetics of the immune response. Unresponsiveness to GLPhe, as examined in this report, involves T_S cell induction as demonstrated by inhibition of GLPhe - CGG responses by GLPhe pre - immunization as well as via the cyclophosphamide experiments. The induction of the T_S cell seems to be a result of a defective antisuppressor pathway as shown by RICA and the functional evaluation of the complexes and the T cells acting as their targets. Although unresponsiveness to GLPhe is due to defects in the antisuppressor pathway, different nonresponder haplotypes may have defects at any one or more stages along this pathway. It is tempting to suggest that it is not necessary to implicate Ir genes for responsiveness and Is genes for unresponsiveness but that both of these states depend on the function of the antisuppressor pathway as the "master switch" of immune responses.

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