TOLERANCE VS. CELLULAR IMMUNITY IN RATS: ROLE OF THE AMINO ACID WITHIN SMALL MOLECULAR WEIGHT ANTIGENS

by

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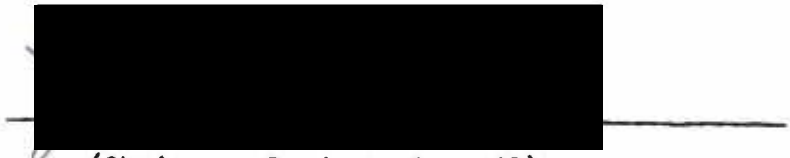

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STATEMENT OF THE PROBLEM

Little is known about the "recognition needs" of the antigen-specific receptor on T-lymphocytes (T-cells) or indeed whether a single receptor type is sufficient to explain the vast variety of T-cell activities. An initial goal of this study was to ascertain the minimum size of the antigen complementary site of the T-cell receptor. Using one of the smallest known complete antigens, Azobenzenearsonate- N-chloroacetyl- L-tyrosine (ABA-T, approximately 450 daltons), we wished to ask; "how much of this molecule contributes to T-cell recognition complementarity"? Unfortunately, we cannot disect out this recognition step for study since, in addition to the specificity of T-cell recognition, antigen must also have present molecular charactoristics, which permit this molecule to initiate an immune response. This step is normally thought to involve "examination" or processing of the molecule by a macrophage-like cell. To pass this initial test, most antigens need a protein component. Tyrosine, in ABA-T, has been shown to provide for this "protein" processing requirement. How tyrosine does provide for the immunogenicity of the antigen is not known. Two possibilities are that either a), tyrosine binds Ia antigens on the surface of antigen-presenting-cells, creating neo-antigens or b), tyrosine triggers antigen-presenting-cells resulting in an allosteric shift of Ia antigens into a new conformation. Following

macrophage triggering, these cells release at least one factor, Interleukin-1 (IL-1). Interleukin-1 provides one of the two required signals for T helper cell activation. The second signal for T helper cells, likely results from the occupation of the T helper cell's receptor specific for the neo-surface structure on the macrophage, either Ia/Antigen units or allosterically transformed Ia antigen. The former hypothesis would require that T cell receptors recognize tyrosine directly or indirectly at the macrophage surface. The latter hypothesis would require clones of T-cells specific for the "active" allosteric state of Ia structure (with no role for ABA-T at this recognition step). T helper cells turned on at this time by both signals, IL-1 directly or indirectly at the macrophage surface, and Ia antigen, would produce and secrete another defined factor, Interleukin-2 (IL-2). Interleukin-2 is known to stimulate other populations of T-cells besides the T helper cell that produces the factor. It is believed that Interleukin-2 provides one of two required signals to T effector cells, the cells that ultimately bring about specific biological function. The second likely signal that T effector cells must receive is occupation of their antigen specific receptor by the apprpriate antigen; in this case ABA-T. Then an effector T-cell would bind the antigen on the surface of the antigen presenting cell. The size of the T-cell receptor for antigen on these cells may or may not permit complementarity with the tyrosine as well as the ABA group. One other possibility is that soluble antigen, not bound to macrophages, in conjunction with

Interleukin-2, could provide the two signals for T effector cell activation.

Since different T-cells may employ different receptor "types" with unique characteristics, we should also ask whether tyrosine is a part of the recognition unit seen by T-suppressor cells. T suppressor cells have been shown not to require antigen presenting cells for their inducement or their expression of suppression. Indeed, several investigators argue that antigen binding to any T-cell free of antigen presenting cells, induces clonal deletion or the induction of suppressor cells. It is interesting to consider, that if antigen presenting cells are not required for presentation of antigen to suppressor cells, then the tyrosine may not be required for either the immunogenicity or specificity of expression of immune suppresor response. In summary, the goals of this study were to examine the role that amino acid specificity plays if any, in both the induction and expression of T-cell function.

INTRODUCTION AND LITERATURE REVIEW

I. THE STILL ELUSIVE T-LYMPHOCYTE ANTIGEN RECEPTOR

In spite of considerable work, identificaton of the T-cell antigen receptor has met with only partial success. Unlike the antigen receptor on B-lymphocytes (i.e. surface antibody), the exact molecular nature of the T-cell receptor is undetermined. Kindred and Shreffler (1972) provided the first evidence that major histocompatability products were crucial for at least T-B cell interactions. Another breakthrough in defining the requirements for the activation of T-cells came when Rosenthal & Shevach (1973), Shevach & Rosenthal (1973), Schwartz et al. (1975), and Schwartz & Paul (1976) demonstrated that T-cell proliferation of immune cells in vitro requires presentation of antigen by accesory cells from the same strain of animal. Important to the understanding of antigen presentation was the finding that presentation of antigen by macrophages to T-cells was genetically linked to the major histocompatability complex, i.e. the H-2 complex in the mouse,.(Benacerraf and Katz, 1975). The first suitable candidates to be identified as immune response (Ir) gene products, were the I-region antigens (David et al., 1973, Hauptfeld et al., 1973). Classic work by Zinkernagel & Doherty (1975) and Klein (1976), provided evidence that Ia antigens (i.e. Class II antigens or

I-region antigens in the mouse) were recognized by T helper cells in context with the foreign antigen. The demonstration that macrophages possess surface I-region products, suggested the possibility that these cells presented foreign antigen in association with Ia products (Erb & Feldmann, 1975 Kappler & Marrack, 1976, Sprent, 1978). The finding, that macrophages with foreign antigen could not induce immune T-cell responses if Ia antigens were blocked on the macrophage surface with a monoclonal antibody to the Ia antigen, supported this conclusion (Baxevanis et al., 1980, Lerner et al., 1980).

Two major theories developed to explain how T-cell antigen receptors could interact with both foreign antigen and histocompatability antigens simultaneously on the surface of antigen presenting cells (Reviewed in Matzinger, P., 1981).

1) The dual recognition theory states that two separate receptors exist on the T-cell. One of the receptors recognizes the foreign antigen while the other receptor recognizes the histocompatability antigen. These two receptors could be coded for by either the histocompatability antigen gene library or the antibody gene library (von Boehmer, H. et al., 1978). Others have suggested that only the foreign antigen receptor is coded for by the antibody gene library and the H-2 receptor is only coded by the H-2 gene library (Bevan, M.J., 1977). A modification of the dual receptor model proposes that there are at least three different antigen receptors on T-cells (Williamson, A.R., 1980). One of the

receptors is the foreign antigen receptor while each of the other two receptors bind either self or foreign H-2 antigens. 2) The altered self receptor theory states that the T-cell receptor is a single receptor which recognizes the H-2 antigen after its modification by foreign antigen. For altered H-2 antigen to occur, it has been necessary to propose that in some fashion, macrophage-like cells process and present foreign antigen in intimate contact with H-2 antigen. Cohn et al. (1978) have argued against this theory since no good evidence exists to support the idea that foreign antigen is linked to H-2 antigen.

Many of the T-cell receptor theories use Jerne's model (1971) to explain how the immune system provides an expanded library to recognize modified self and possibly foreign antigens as well. Jerne and his collegues (von Boehmer, et al., 1978) later refined his original model and proposed that two anti-self receptors are initially present on the immature T-cells. One type is retained while the other anti-self receptor type has to undergo rapid modification in order for the cell to survive tolerance, leading to the generation of receptors for foreign antigen. A role for "somatic mutation" in the generation of the T-cell antigen receptor library has not as yet been documented.

In 1975, Binz and Wigzell (1975a) provided the first suggestive evidence that T-cell antigen receptors possess antibody idiotypes. Using anti-idiotype antibody these authors were able to purify the "T-cell" antigen receptor (Binz & Wigzell, 1975b, Binz & Wigzell,

1976). With anti-idiotype antibody and the appropriate genetic analysis, these and other authors were able to provide the first evidence that T-cells have detectable V-H gene markers, suggesting T-cells use the B-cell V-H gene library (Rajewsky, et al., 1977, Cosenza, et al., 1977, Krawinkel, et al., 1977, and Eichmann, 1978). In addition, several authors have reported that T-cell responses specific for some antigens, are linked to specific alleles of the Igh-1 locus which codes for immunoglobulin constant regions. In support of this, Weinberg, et al. (1979) found that mice could be primed to express delayed hypersensitivity (DTH) with anti-cross reactive idiotype (CRI) antibody. Secondly, they asked if T-cell receptor characteristics mimic those of the B-cell. To ask this, they employed a unique strain of mice which produces antibody that binds NP haptens better then the NIP hapten cunjugate originally used to desensitize the animals. This "heteroclitic" response has been linked to expression of their $Igh\text{-}1^{b}$ gene. They then asked if T-cells displayed a similar "heteroclitic" specificity. Their finding that T-cells do display a heteroclitic reaction, and that such reactivity was indeed linked to the Ig gene region, that is the IgH-1 locus, suggested that T-cells share Ig gene products during antigen recognition. In a similar finding Jayaraman and Bellone (1982) found that low levels of anti-idiotype antibody induced DTH reactivity; whereas, higher levels suppressed DTH reactivity. Sugimura et al., (1981) found that anti-T15 idiotypic antibody blocked both T-effector cells and T-suppressor cells in CBA/N mice

primed to phosphorylcholine (PC). In partial support that the antibody library may be used by T-cells, Forster et al. (1980) suggested that detectable alterations of DNA sequences adjacent to the C-mu gene in cloned T-cell lines could be interpreted as evidence that T-cells use the B-cell V-H gene library.

Owen et al., (1979, 1980, 1981, 1982, & 1983), recently reported detecting potential allotypic determinants on "constant regions" of the T-cell receptor (IgT-C region). This research group argues that these determinants (Tthy, Tind, Tsu, and Tpre) are present on various T-cell sub-populations, appearing in an ordered sequence during differation and ontogeny. It is yet to be established whether these determinants are part of a putative constant region on T-cell receptors.

However, the evidence that the T-cell antigen receptor shares the B-cell V-H gene library has been subjected to considerable criticism, recently reviewed by Jensenius and Williams (1982). Careful examination reveals no T-cell DNA V-H gene rearrangement (Kronenberg et al., 1980a, Cayre et al., 1981 & Bleackley et al, 1982) or evidence of T-cell mRNA for either V-L or V-H gene expression (Kronenberg, 1980b & Kemp et al., 1982). Also, some "anti-idiotype antibodies" were found to be specific for cross-reacting carbohydrates, (Layton, J.E., 1980, Mattes, M.J. & Steiner, L.A., 1978), rather than being directed against the idiotype. Recently, it was found that what looked to be a promising candidate for the T-cell receptor, a T-cell hybridoma factor that

bound hapten and was Igh-1 idiotype positive, was later found to be produced by non-lymphoid cells (Clark, & Capra, 1982).

II. THE REQUIREMENT FOR MACROPHAGE-LIKE CELLS TO PRESENT ANTIGEN

As indicated earlier, foreign antigens are usually presented by macrophages or macrophage-like cells before T-cells can respond to these foreign antigens. This event is commonly referred to as the "processing and presentation" of antigen for T-cells. Hersh and Harris (1968) demonstrated the need for macrophages to be present for the elicitation of T-cell proliferation to antigen stimulation. It was also found by these investigators, that intimate contact appeared to be required for the elicitation of T-cell proliferation. Macrophages were shown to play an intimate role in the presentation of antigen to T-cells by the work of Waldron et al. (1973). Rosenwasser and Rosenthal (1978) showed that xenogenic macrophages or fibroblasts could not present antigen to T-cells successfully. They showed that macrophages from the same species did work as expected. These findings have been confirmed for several species, i.e. in guinea pigs (Thomas et al., 1977), mice (Cowing, et al., 1978, Kramer, et al., 1980), and humans (Hirshberg, H., 1978, Bergholtz, et al., 1979).

III. REQUIREMENTS FOR SUCCESSFUL PRESENTATION OF ANTIGEN BY

MACROPHAGE TO T-CELLS

A. Genetic restriction:

Genetic restriction refers to the finding that for T-cell / macrophage interaction to occur, not only must macrophages and T-cells be from the same species, but they must also be homozygous at certain loci of the major histocompatability complex. Major work done by many investigators, both *in vivo* (Green et al., 1967, Zinkernagel, 1974, Zinkernagel, et al., 1978, Miller et al., 1975, and Miller et al., 1976) and *in vitro*, (Rosenthal and Shevach, 1973, Shevach and Rosenthal, 1973, Shevach et al., 1972, Thomas and Shevach, 1976, Thomas, 1978, Erb and Feldmann, 1975, and Yano, et al., 1977), have shown that T-cell immune responses to proteins or hapten-conjugated-proteins require I-region homology between the presenting macrophage and the responding T-cell. In addition, T-cell responses are prevented by the addition of antiserum or monoclonal antibody specific to I-region products both during primary, (Thomas and Shevach, 1976, Thomas, 1978, Ford, et al., 1982) and secondary responses, (Baxevanis et al., 1980, Lerner, et al., 1980, Schwartz, et al., 1976, Schwartz, et al., 1978, Hodes, et al., 1980, Ford, et al., 1982).

B. Other requirements:

Investigations examining which macrophage types work best in assisting in immunological responses indicate that small immature macropages are best for the presentation of antigen (Lee, et al., 179, Tzehoval, et al, 1981). In addition during antigen presentation, macropages that display both parental Ia antigen phenotypes work better than macrophages that only display one shared Ia antigen on their surfaces for secondary responses (Berle and Thorsby, 1980, Thorsby, et al., 1982, Ford, et al., 1982). Metabolically active macrophages appear to be required since metabolically inhibited macrophages worked less efficiently then metabotically active macrophages in the presentation of antigen (Ziegler and Unanue, 1981). Considerable evidence supports the finding that physical contact is required between macrophages and T-cells during cellular interactions leading to immunological response (Salvin and Nishio, 1969, Salvin, et al., 1971, Hanifin and Cline, 1970, Werdelin et al., 1974). Even though T-cells can bind to macrophages in an antigen-independent manner, (Lipsky and Rosenthal, 1975, Lipscomb, et al., 1977, Ziegler and Unanue, 1979), successful, antigen-dependent binding requires that a) the macrophage present appropriate antigen, and b) the macrophage share major histocompatability gene products with the immune T-cell (Lipsky and Rosenthal, 1975, Ziegler and Unanue, 1979, Braendstrupet al., 1979).

IV. WHAT ANTIGENS INDUCE STRONG T-CELL IMMUNITY?

Studies determining which molecules could work as successful antigens in the development of cell mediated immunity, indicated that proteins in most cases play an essential role (Hay, 1979). Polysaccharides and oligsaccharides are considered to be poor immunogens of DTH (Gerety, et al., 1970). Schneider and de Weck (1967) reported success in sensitizing guinea pigs with penicilloyl-dextran. Gerety, et al. (1970) were successful in inducing and eliciting DTH response to pneumococcal polysaccharides. When monosaccharides, disaccharides or oligosaccharides are conjugated to amino acid polymers or polypeptides, "hapten specific" immunity to the sugar has been demonstrated (Borek, et al., 1963, Tremaine, 1963). Other material studied for possible T-cell antigens include lipids. Lipids by themselves are believed to be in most cases non-immunogenic (Rapport and Graf, 1969), but when attached to proteins or small molecular weight antigens, may increase the immunogenicity of a molecule or even become "haptenic determinants" (Arnon, et al., 1969, Matterson and Leskowitz, 1977) for antibody production or DTH. Except for the two first studies above, the molecular requirement of a protein component in T-cell antigens, suggests that polysaccharides lack the necessary requirements provided by proteins for imunogenicity (Borek et al., 1963).

Of great interest to many investigators has been the use of synthetic amino acid polymers or proteins that are or are not

conjugated with defined chemical haptens. The use of haptenated polymers have been used to define some of the size requirements for the successful immunogenicity of these molecules. Schlossman et al., (1965) found using DNP conjugated poly-lysine, that immunogenicity was lost when less than seven lysine amino acids were used. Work done by Gell and Benacerraf (1961), Benacerraf and Levine (1962), Gell and Silverstein, (1962), and Silverstein and Gell (1962), found that immunizing with hapten modified protein would lead to a immunodominate response against carrier (i.e. protein) determinants and not to the haptenic determinant. Leskowitz (1963) was the first to successfully show immunodominant T-cell responses to a haptenic determinant. He used azobenzenearsonate (ABA) conjugated to poly-L-tyrosine, and showed that the immunological specificity was to the haptenic epitope. Two years later, Leskowitz (1967) determined that in guinea pigs, ABA-N-acetyl-L- Tyrosine (ABA-T) could act as a complete antigen requiring no more than one amino acid for immunogenicity. Because of the unusual nature of this response, ABA- conjugates of polymers, proteins, and single amino acids, have been used extensively analyze the structural components required for immunogenicity.

Much remains undetermined for this system; i.e., "why are proteins or peptides, and in the special case of the ABA hapten, single amino acids, necessary for the immunogenicity of most T-cell responses"? What role does the amino acid conjugated with haptens play in the specificity of T-cell responses?

V. ABA-T AND OTHER ABA- CONJUGATES

1. CARRIER REQIREMENTS

Since 1963 when Leskowitz first described "hapten specific" responses in guinea pigs sensitized to ABA-poly Tyrosine, interest in ABA and related molecules has grown. As previously discussed, most DTH responses are specific to the "carrier" portion of a hapten-carrier antigen. Finding that ABA conjugated to a number of poorly immunogenic molecules induced anti- ABA DTH commanded great interest and study. That these "poorly" immunogenic molecules provided some essential component to the response, is supported by the finding that ABA conjugated to a number of D-amino acid polymers were not immunogenic (Benacerraf, 1963, Leskowitz, et al., 1966, Jones & Leskowitz, 1966, Collotti & Leskowitz, 1970, Bullock et al., 1975a). Borek, et al. (1967) reported that ABA- conjugated D-amino acid polymers were immunogens, but animals required twice as long to become sensitized. In other studies with ABA conjugated to various proteins, investigators found that if ABA was conjugated to strong immunogenic proteins, the specificity of a DTH response was primarily directed to the "carrier" determinants (i.e. the protein was immunodominant), (Jones & Leskowitz, 1965, Schwartz & Leskowitz, 1969, Bullock, et al., 1975b). But if ABA-T was mixed with, but not linked to an immunodominant protein, strong response were obtained

to both ABA-T and protein epitopes (Schwartz & Leskowitz, 1969). However, it was later observed that in fact, a large molecular weight antigen was unnecessary for this unique molecule. ABA conjugated with either a single D- amino acid (Leskowitz, et al., 1966) or ABA conjugated with a single L - amino acid worked well (Jones & Leskowitz, 1965, Leskowitz, et al., 1966).

Some studies have suggested that polypeptides or single amino acid "carriers" are not only essential for sensitization, but are also critical for tolerance induction. Thus, ABA- D- polymers, di-ABA- N- acetyl tyrosine or heavily conjugated poly- L- tyrosine failed to induce either immunity or tolerance (Collotti & Leskowitz, 1970, Bullock et al., 1975a). The conclusion of these investigators was that the carrier dictated how an antigen was manipulated. Most importantly, these studies failed to indicate whether the carrier molecule contributed to the specificity or processing of antigen during the initial steps of tolerance.

2. ROLE OF MACROPHAGE IN PRESENTING ABA-T

As discussed previously, antigen presenting cells in most cases, are essential for the development of T-cell immunity. Positive evidence for the requirement of macrophage with ABA-T is incomplete. However, suggestive evidence indicates that Ia^+ antigen presenting cells do play an essential role in the development of T-cells responding to ABA-T. It was found that modification of

ABA-T with the addition of increasing lengths of lipid tails, decreased the immunogenicity of the molecule when studied with an *in vitro* DTH assay (Mattern & Leskowitz, 1977). In contrast, it was found that if macrophages from a non-sensitized syngeneic animal were pulsed with either ABA-T conjugated to a small lipid tail or a long lipid tail, better stimulation of ABA-T primed T-cells occurred using the longer tailed molecule. The authors concluded that this was due to the greater "stickiness" of the longer molecule. This suggested that if the antigen could be altered in such a manner that made it more accessible for macrophage uptake, a stronger state of sensitization was achieved. Finally, the authors showed that depletion of macrophages from primed *in vitro* cultures eliminated any response to the ABA-T - lipid conjugates; and, adding back fresh macrophages restored the response. This result strengthened the proposal that macrophages are important for the presentation of ABA-T to immune T-cells.

Lawn & Leskowitz (1980) showed that Ia^+ cells are important for the expression of immune T-cells. They found that anti- Ia antisera blocked a secondary responses *in vitro* by ABA-T primed Lewis rats lymphocytes. Thus, it appears that the response to ABA-T challenge in rats requires presentation of antigen in context with Ia antigen. Recent work by Bhan and Leskowitz (1982) examined differences between macrophages conjugated with the activated diazonium salt of ABA vs. pulsing macrophage with the complete, non- reactive molecule ABA-T. These authors found that although guinea pigs could be

primed with either ABA-T -pulsed-macrophages or ABA- conjugated-macrophages, distinct differences were found in the specificity of DTH response. Only animals primed with ABA- conjugated-macrophages developed responses when challenged with ABA- conjugated-macrophage. These animals failed to respond to ABA-T pulsed macrophages and responded poorly to ABA-insulin challenge. In contrast, when ABA-T pulsed macrophages were used to prime animals, ABA-T -pulsed-macrophages elicited strong skin reactions, whereas, ABA-conjugated macrophages failed to elicite DTH reactions in these animals. Such animals also responded to ABA-insulin challenge. When primed with ABA-T/CFA, the animals developed stronger reactions with ABA-T pulsed-macrophages than when challenged with ABA- conjugated-macrophages. This work suggests that the use of conjugated syngeneic cells may expand a majority clone of T-cells responsive to modified histocompatpability determinants rather than the ABA- amino acid conjugate.

3. IMMUNOGENICITY VS. SPECIFICITY: THE UNANSWERED QUESTION

In studying the specificty of guinea pig response to ABA-L-polymers, Leskowitz, et al. (1970) found that animals produced larger DTH skin reactions when challenged with ABA polymers possessing some of the original sensitizing amino acid. But responses could also be evoked with polymers that had none of the original sensitizing amino acids, even though skin reactions were

smaller. Several laboratories followed up on these initial studies by synthesizing a variety of ABA-T analogues. They then asked, "which elements of this small molecule are central to specificity and which regions or characteristics are essential for recognition and processing by macrophages, i.e. imunogenicity"? Progressive elimination of the alpha- amino end of ABA-T showed that elimination of either the amino or the carboxy group diminished the immunogenicity of the molecule while elimination of both groups completely destroyed the molecules immunogenicity (Alkan, et al., 1972, Bush, et al., 1972, Hanna & Leskowitz, 1973). Removal of the alpha carbon but retention of both the carboxy and amino groups or just one, yielded an imunogenic molecule (Alkan et al., 1972). It was concluded by Alkan, et al. that some "polarity" was required at the alpha- amino end of the amino acid for immunogenicity. Hanna and Leskowitz (1973) found that when these modified molecules were studied as challenging antigens <u>in vitro</u> with ABA-T immune guinea pig lymph node cultures, molecules lacking the carboxy group were more efficient in stimulating blastogenesis than molecules lacking the amino group. These investigators also found that the molecule lacking both amino and carboxyl groups, failed to elicite any blastogenic response. It should be noted that the later group reported that ABA-T, which did not have the blocking acetyl group on the amino group, was an extremely poor sensitizing antigen and challenging antigen. This is in contrast with the earlier group of investigators who routinely used the "acetyl free" ABA-T molecule

successfully as a sensitizing antigen (Alkan,et al., 1972, Bush, et al., 1972, Bellone, et al., 1975).

It must be remembered that some of the previous studies in guinea pigs suggested that the amino acid in these small ABA- amino acid conjugates was not only important for the immunogenicity of the antigen, but may also play some role in the specificity of response. As mentioned above, animals immunized to ABA- polymers responded better to challenge when the challenging polymer contained some of the original amino acids (Leskowitz, et al. 1970). Later work showed that using an in vitro macrophage inhibition assay (MIF), the apparent amino acid contribution to specificity of response increased with time after initial sensitization. These results were obtained in both the guinea pig and rat model (Jokipii, et al., 1975a, Jokipii, et al., 1975b). These authors claimed that initially, the response was dominant for the "hapten" ABA rather that for the "carrier" amino acid. As time progressed from immunization, there was a marked increase in MIF when cells were callenged with the original ABA- amino acid conjugate versus the use of another ABA- amino acid. A recent preliminary experiment reported by Lawn & Leskowitz (1980), indicated that Lewis rat peritoneal exudate cells could show preferential blastogenic responses to the original priming ABA- amino acid antigen rather than to another ABA- amino acid antigen although, some cross-reactivity was present.

It must be emphasized that ABA- conjugated proteins present

both ABA- determinants and neo- protein determinants resulting from conjugation with ABA. Usually neo- protein determinants are immunodominant. This was eloquently demonstrated by Ray and Ben-Sasson (1979), when these authors showed that animals immunized to ABA-protein responded to both ABA- conjugated to guinea pig albumin (GPA) and p-Azobenzoic acid (ABS)- conjugated to GPA, i.e. similar neo- antigens but distinct haptens. Whereas, it had previously been shown that when ABA and ABS on single amino acids were used as the sensitizing antigens, the animals responded preferentially to proteins conjugated with the original hapten for the expression of DTH (Alkan, et al., 1972). Neo- protein determinants are so potent that when Ray and Ben-Sasson (1979) immuninized guinea pigs with ABA- conjugated to GPA, a self protein to which they are tolerant, challenge in vitro with ABA-T induced only poor responses while the neo- antigens of ABA-GPA or ABS-GPA produced strong responses. However, when guinea pigs were primed to ABA-T, i.e. no neo- protein determinants, animals produced only mild responses to the ABA-GPA but strong responses to free ABA-T. The authors concluded that ABA-conjugated to the poorly immunogenic protein GPA, generated determinants uniquely different from the ABA- amino acid determinant recognized by guinea pig T-cells sensitized with the amino acid conjugate. These results may explain the results of Bhan & Leskowitz (1982) that ABA- conjugate macrophages sensitized different T-cell clones than did macrophages pulsed with ABA-T, since conjugated macrophage would have ABA- conjugated to "self"

proteins (see above). Thus, ABA-T pulsed-macrophages would present a different epitope from the epitope presented on macrophage conjugated with ABA on their surface.

Even though much has been learned by using ABA- conjugated proteins, polypeptides, and single amino acids, there is lack of knowledge about what the exact antigenic determinants are on these various molecules. That is, what portions of these various molecules are important for interacting with the T-cell antigen receptor? Using single amino acid conjugates should provide some answers as to what exactly is the antigenic determinant in these simple molecules. Unanswered questions include what role does the amino acid play in both the specificity and immunogenicity of these simple molecules. Do different subpopulations of T-cells recognize the same determinant on these amino acid conjugates, or do different determinants interact with different T-cell subpopulations? What role does the amino acid play with different subpopulations of T-cells? Answering these questions will provide greater insight into what are the T-cell receptor requirements for the expression of T-cell functions.

VI. NEUTROPHIL AGGREGATION AND SUPEROXIDE GENERATION: DEVELOPMENT OF A NEW ASSAY FOR THE DETECTION OF ANTIGEN- INDUCED LYMPHOKINE RELEASE WITH RATS.

Two *in vitro* assays have been used extensively to measure antigen specific, T-cell mediated immune function: measurement of antigen-induced blastogenesis by labeled thymidine uptake, believed to measure T-cell activity (Oppenheim, 1968), and measurement of Macrophage Inhibition Factor (MIF) production as indicated by cell migration. The major advantage of the latter assay over the former is that the MIF assay essentially measures a biological event directly relevant to the actual expression of immunity. The major problems of the MIF assay are the tedious protocol involved in detecting the presence of the lymphokine and the difficulty in obtaining a sufficent number of cells for the assay. Blastogenesis, though an *a priori* event before the expression of T-cell immunity, can occur for a number of reasons not dependent on antigen inducement. However, a major advatage of the blastogenesis assay is that large numbers of cultures can be established in any given experiment, allowing for more experimental questions and greater statistical reliance. An ideal assay would measure lymphokine release due to immune stimulation using low numbers easily obtained cells. With these goals in mind, neutrophils were chosen as potential candidates to wse in an immune assay.

The role of neutrophils in T-cell immunity is still poorly defined. Toffaletti et al. (1980) provided evidence that PPD induced release of MIF occurs without macrophage and only in the presence of neutrophils. It is known that T-cells release several lymphokines that affect neutrophil activity. One of the better

characterized lymphokine in this regard is leukocyte inhibitory factor (LIF), a factor that works essentially in the same fashion as MIF (Dynesius-Trentham et al., 1981). The production of LIF has been shown to parallel antigen induced *in vitro* blastogenesis and *in vivo* DTH responses in the rat (Trentham, et al., 1978, Trentham, et al., 1980). However, the measurement of LIF is as tedious as the measurement of MIF. The development of a rapid quantitative assay measuring the aggregaton of neutrophils, an event believed to reflect neutrophil margination on vascular walls just prior to leaving the vasculature (Egger, et al., 1982), has been recently reported in our laboratory, (Bullock, et al., 1982). The application of this assay to rats is described here. This assay simultaneously measures super-oxide generation, produced when neutrophils undergo an "oxidative burst", as assayed by the reduction of nitroblue tetrazolium (Baehner & Nathan, 1968, Newburger, et al., 1979, DeChatelet, 1978).

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CELL MEDIATED IMMUNITY IN LEWIS RATS SENSITIZED TO ABA-T. I. VARIATIONS BETWEEN *IN VIVO* AND *IN VITRO* ASSAYS

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ABSTRACT

In vivo DTH skin reactivity and *in vitro* induced blastogenesis are frequently presented as representing a common underlying T-cell mechanism. We therefore asked whether these phenomenon have similar sensitization and elicitation antigen requirements, similar kinetics of appearance, and similar antigen specificities. To answer these questions, we chose the small, completely defined antigen ABA-T because it induces potent cell mediated immunity while failing to induce significant antibody production. The Lewis rat was chosen because it yields both strong classic DTH reactions and large stimulation indices *in vitro*. We report here that these two immune activities differed in 1) the optimum site for sensitization, 2) the range of antigen dose yielding strong reactions, and 3) the kinetics of the appearance of maximum response. We found that animals receiving either footpad or intra-peritoneal (IP) injections of ABA-T were equally capable of developing strong DTH responses. However, only the IP route of immunization resulted in strong spleen cell blastogenesis. Animals could be primed for DTH responses within a broad range of antigen sensitization doses, 40-2000 mcg ABA-T/IFA; whereas only 50-100 mcg ABA-T/IFA per animal resulted in meaningful levels of antigen-induced blastogenesis. Finally, maximum DTH responses were obtained 10-14 days after priming. This early period was clearly distinct from the longer 20 day period

required to obtain maximum antigen-induced blastogenesis with spleen cells from IP primed animals.

INTRODUCTION

When studying cell mediated immunity, *in vivo* measurements should provide the most reliable indicators of the true status of host cell- mediated immunity (CMI). Unfortunately, this approach entails several drawbacks such as the requirement for large numbers of animals to insure significant data and the difficulty in manipulating such *in vivo* systems to detect and control subtle interactions of various cells with each other and with factors required for an immune response. Therefore, several *in vitro* assays have been developed to overcome these limitations of *in vivo* analysis (Nauciel & Raynaud, 1971, Becker, et al., 1973, Wunderlich & Canty, 1971, David, 1971). *In vitro* approaches require the major assumption that *in vitro* analysis truly reflects *in vivo* activity. Unfortunately, this is a difficult assumption to verify, with few investigators reporting detailed comparative studies of *in vivo* and *in vitro* analyses. This paucity of data is particularly apparent with the studies of rats (Jokipii et al., 1975, Sobel, et al., 1975, Lawn and Leskowitz, 1980, Bullock, 1978). Since the use of laboratory rats for immunological studies is increasing, due in part to the enlarging numbers of congenic strains, we decided as an initial step of our study of CMI in rats to compare *in vivo* DTH skin

reactivity to *in vitro* blastogenisis. *In vivo* and *in vitro* reactions were compared with respect to the effects of the route of antigen administration, antigen dose requirements, role of adjuvant, the kinetics of development, and the specificity of the resulting immune response. For this study, we used the simple and well characterized molecule, azobenzenearsonate -N-chloroacetyl -L-tyrosine (ABA-T), which induces potent cell-mediated immunity, in the absence of a significant antibody response (Collotti & Leskowitz, 1970, Becker, et al., 1975, Sobel, et al., 1975). With this system, rats react with both strong, classic skin reactions of 15-20 mm diameter and up to 40 fold antigen stimulation indicies following spleen cell culture.

We report here that the route of antigen administration for sensitization was critical for the blastogenesis assay but not for skin reactivity. Maximum skin reactivity occurred 8-10 days before maximum spleen cell blastogenesis was achieved. When the *in vitro* assay was used, marked variation in individual animal responses were observed, with some animals failing to respond, whereas DTH skin reactions were largely uniform. Finally, though profound differences between the two assays existed, the antigenic specificity of both assays remained the same.

Materials and Methods

Rats: Lewis female rats were purchased from either Jackson Laboratories, Bar Harbor, Maine, or Microbiological Assoc., Inc., Walkersville, Maryland. In some experiments, Lewis female rats were from our breeding stocks purchased from the above sources.

Chemicals: p-Arsanilic acid was purchased from Eastman Kodac Co., Rochester, New York. N-chloroacetyl-L-tyrosine and human gamma globulin (HGG), Cohn Fraction II used to prepare ABA-59-HGG and ABA-3.6-HGG were purchased from Sigma Chemical Co., St. Louis, Missouri. HGG used in preparing ABA-39-HGG was purchased from Pentex Inc., Kankakee, Illinois. Hen egg ovalbumin (Oval) used to prepare ABA-6-OVAL was purchased from Pentex Inc., Kankakee, Illinois.

Preparation of antigens: Preparation of ABA-T and ABA-proteins has been described previously (Bullock, 1978). The procedure is a modification of the method described by Tabachnick and Sobotka (1959). Briefly, the diazonium salt of p-arsanilic acid was conjugated to the chloroacetylated amino acid tyrosine to form

p-azobenzenearsonate- N-chloroacetyl- L- tyrosine (ABA-T). ABA-proteins were produced in a similar manner and dialyzed extensively against normal saline. ABA-T, due to its small molecular weight, could not be dialyzed and instead was acid precipitated and washed three or four times. Both ABA-T and the ABA-proteins were resuspended in Sorensen's phosphate buffer saline (PBS) and the pH brought to 7.2-7.4 with 1N NaOH. Protein concentrations were determined by the Lowry method (1951) and both the degree of ABA substitution on the proteins and the concentration of ABA-T were determined spectrophotometrically. The ABA component was determined assuming an extinction coefficient of :

E^{1M} = 10,500 at 490nm in 0.1N NaOH.

Immunization: All rats received 0.1 - 0.5ml Equi-Thesian (prepared in our laboratory) intraperitoneally (IP) as anesthesia prior to all procedures. Antigens for injection were prepared by emulsifying equal volumes of antigen in PBS in either incomplete Freund's adjuvant (IFA) (Difco Labs, Inc.) or complete Freund's adjuvant (CFA) with Mycobacterium butyricum or M. tuberculosis H37RV (both from Difco Labs, Inc.). Animals were given 0.2ml of the antigen emulsion either IP or equally distributed into the four footpads (FP).

Induction of DTH: Animals were anaesthetized and their sides shaved. Intradermal injections (ID) of 0.1ml volumes containing the test antigen dissolved in PBS were administered up to 3 sites per side since multiple injection sites were found not to significantly affect reaction diameters. The diameter of induration and erythema was measured (mm) after at least 24 hours. When both induration and erythema were present, their reaction diameters were found to be comparable. If only induration or erthema was present, then this measurement was recorded as the reaction diameter. Arthus-like reactons were not observed.

In vitro spleen cell culture: Animals were anesthetized and exsanguinated prior to removal of spleens. The animals were sacrificed by cervical separation and the spleens were removed. Spleen cells were teased out into ice cold Eagle's Minimal Essential Medium, with Earle's salts and Hepes buffer (MEM, Flow Labs., Inc., Inglewood, CA). Cells were washed 3 times in cold MEM and then counted with a hemocytometer. The complete culture medium contained, in addition to MEM, 10 mM nonessential amino acids, 100mM Na Pyruvate, 200 mM L-Glutamine, 2.0 mg/ml Na bicarbonate, 100 units/ml penicillin, 100 mcg/ml streptomycin, 2.0×10^{-5} 2-mercaptoethanol and 5% heat-inactivated fetal calf serum (FCS, Flow Labs., Lot # 4056012). The complete culture medium was

typically made in 100ml batches or multiples thereof on the day of use. Cells were cultured at 2.0×10^6 cells/ml in a final volume of 0.25 ml per well in Falcon Micro-Test II plates maintained in 5% CO_2 and 95% air atmosphere at 37^o C. Cells and antigens or the mitogen concanavalin A (Con A, Pharmacia, New York) were all diluted in complete culture medium prior to addition to culture wells. All experiments included Con A stimulated positive controls. In all experiments, Con A at its optimal concentration of 5 mcg/ml, elicited a response of >= 1.0×10^5 counts per minute (CPM) of incorporated ^{3}H- Thymidine. To assess stimulation, 0.5mcCi ^{3}H-Thymidne (specific activity 20-25 Ci/mM, NEN) was added to each culture one day prior to harvesting. All cultures were maintained for six days. Cells were harvested onto glass fiber filters, dried and counted in Insta-gel (Packard Scientific) or a Omnifluor (NEN) - toluene (Baker) scintillation cocktail using a Searle Scintillation counter. Vials and pads without cells in scintillation cocktails gave an average of 50 CPM as background and were not subtracted out for calculations.

Statistics: For the <u>in vivo</u> DTH assay, each group contained 5-7 animals. The <u>in vitro</u> blastogenesis assay used 6 cultures of cells, pooled from 2-4 animals except where indicated, per antigenic

or mitogenic dose. Usually 12, though sometimes six unstimulated cultures were used for negative controls. Means and the standard error of the mean (SEM) were calculated arithmetically. Statistical significance was calculated by using the Mann-Whitney U test. Probabilities of 0.05 or less ($p <= 0.05$) were considered statistically significant. The maximum stimulation index is shown in parentheses. The maximum stimulation index was calculated as:

$$\frac{\text{Maximum CPM obtained for antigen used}}{\text{Maximum CPM of cells not challenged with antigen or mitogen.}}$$

RESULTS

RELATIVE DTH RESPONSIVENESS OF RAT STRAINS TO ABA-T ANTIGEN

To optimize our DTH model, we first chose to determine which strain of rats was most suitable for our studies. For that purpose, rats from eight different strains were injected with 50mcg ABA-T/IFA in the footpads. The animals were rested 14 days and then challenged intradermally (ID) with 50mcg ABA-32-HGG. Skin reaction diameters were measured 24 hours later and the results are displayed in Figure 1. Of the eight strains tested, seven were positive. Of the seven positive strains, one strain, the Lewis strain, produced the most intense skin reactions. The remaining six, though displaying positive reactions, produced consistently poorer responses. Only the ACI strain failed in this and subsequent studies to elicit any DTH response to ABA-T priming. ACI animals did respond to PPD skin testing following injection of ABA-T/CFA in subsequent experiments but still failed to respond to ABA-T.

Female rats were chosen as our test animals on the basis of these and earlier studies from our laboratory indicating that females responded much earlier and more strongly than males.

EFFECT OF ANTIGEN PRIMING DOSE ON INTENSITY AND PERSISTANCE OF SKIN

REACTIONS

We next wished to determine both the optimal priming dose and the optimal time to measure the skin reactions as they developed in immune animals following challenge with the appropriate antigen. Five groups of Lewis adult female rats were injected with either IFA or 2-500 mcg of ABA-T/IFA via footpads as indicated in Figure 2. All animals were challenged 14 days after immunization and skin reactions were measured on the same animals at 24 and 48 hours post-challenge. The results in Figure 2 demonstrate that animals which had received 50mcg or more of ABA-T produced essentially the same intense skin reactions when measure at 24 hours post-challenge. However, the diameters of the reactions diminished noticeably within the next 24 hours, except for the group of animals that had received a high, 500mcg ABA-T priming dose. Animals receiving less than 50mcg ABA-T displayed either poor or no response to skin challenge.

ROLE OF BACTERIAL ADJUVANTS DURING RAT SENSITIZATION TO ABA-T

When guinea pigs were injected with ABA-T emulsified in IFA, rather than inducing immunity, strong tolerance was established (Bullock, et al., 1975). In contrast, Lewis rats primed with ABA-T

in IFA were not tolerized but instead developed strong immunity as shown above. However, as previously shown (Bullock, 1978), animals which responded to primary ABA-T/IFA injections, were refractory to secondary ABA-T injections 49 days later. This suggests that tolerance is a natural sequel to primary immune responses in ABA-T/IFA sensitized rats. It was therefore of interest to see if even larger reactions could be induced by giving ABA-T emulsified in CFA. Two different Mycobacterium-containing emulsions were prepared with ABA-T as well as an IFA emulsion containing only antigen. Three groups of animals were injected with 50mcg ABA-T using one of the three emulsions. Animals were skin tested 14 days later with either 50mcg ABA-6-Oval or 10mcg PPD. As shown in Figure 3, rats primed with ABA-T/IFA, in contrast to the results obtained with guinea pigs primed with ABA-T/IFA , produced very strong reactions. Only slightly larger responses were seen in animals injected with ABA-T/H37RV-CFA while ABA-T in M. butyricum adjuvant actually produced similar or slightly smaller reactions than these obtained with IFA only.

Recently it was reported that ABA-T/IFA failed to sensitize Lewis rats (Lawn & Leskowitz, 1980). Animals so primed failed to respond to skin test challenge with ABA-Insulin. We obtained strong responses following ABA-T/IFA priming when either ABA-HGG or ABA-Oval were used as the challenging antigen (Figures 1-3,5-8) but we had not tested ABA-T/IFA primed Lewis female rats with ABA-Insulin. We therefore skin tested ABA-T/IFA primed Lewis female

rats with ABA-4-Insulin using the 20mcg dose used by Lawn & Leskowitz, and a 100mcg dose (Figure 4). As shown, 20mcg was in fact, an insufficient amount to induce significant DTH. When the challenging dose was 100mcg, strong skin reactivity was induced. As Lawn & Leskowitz had suggested, it appears the challenging dose they had used was insufficient to elicite DTH reactions.

EFFECTS OF ANTIGEN DOSE AND TIME AFTER SENSITIZATION ON THE DTH RESPONSES

To determine the optimum time after sensitization to elicite DTH reactions, adult female rats were immunized in the footpads with IFA only or 2, 10, 50, 250, or 500mcg of ABA-T/IFA. All animals were then challenge on days 7, 10, and 21 post-priming. The challenge antigen used was 50mcg ABA-16-HGG in PBS (previously shown to be an optimal challenge dose) given intradermally. Skin reaction diameters were measured 24 hours after skin testing. As shown in Figure 5, the largest response diameters occurred 10 days after animals were immunized with ABA-T/IFA. Equally strong reactions were obtained with priming doses of either 50, 250, or 500mcg ABA-T per animal. When less than 50mcg ABA-T/IFA per animal was used, either poor or no responses were detected. Animals challenged 7 days post-priming presented a similar but smaller response curve compared to day 10. A lesser degree of responsiveness was seen at

21 days post-priming. Those animals that were immunized with the very high 500mcg ABA-T/IFA dose gave especially poor responses to skin challenge at 21 days, suggest an exhaustion or feedback suppression resulting from this massive dose.

The persistence of limited sensitivity over much longer periods of time is indicated in Figure 6. Again Lewis female rats were primed with 50mcg ABA-T/IFA in the footpads. The challenging antigen used was 50mcg ABA-16-HGG/site given on various days after priming as shown. The solid line represents animals that received antigen in IFA while the dotted line represents animals that received only IFA. As was noted in Figure 5, optimal responses occurred at days 10 and 14. We have consistently observed that skin challenge from 10 to 14 days post-priming yields maximal and essentially equivalent responses. However, in contrast to guinea pigs, after 14 days, the intensity of DTH reactions in rats diminishes rapidly.

SENSITIZATION FOLLOWING ANTIGEN ADMINISTRATION, INTRAPERITONEAL VS. FOOTPAD

We next compared routes of administration, hoping to discover a convenient route of injection which might establish a state of tolerance. Intraperitoneal (IP) injection was chosen as a possible route and a comparative study of footpads versus IP injection was

conducted. Six groups of adult female Lewis rats received either an injection of IFA only or from 8-2000 mcg ABA-T/IFA equally distributed in four footpads or given at one site intraperitoneally. Ten days later, all animals were skin tested with 50mcg ABA-11-Oval/site and 24 hours later the reactions were scored. Figure 7 displays the results of this experiment. Based on skin testing, we found that both modes of injection established comparably strong immunity. As little as 8mcg ABA-T was able to induce immunity albeit suboptimal. However, higher doses from 40-2000 mcg ABA-T resulted in strong immunity with administration of the antigen via either IP or FP injection. Essentially no qualitative differences were detectable for the DTH reactions by either priming route.

ONTOGENY OF IMMUNOCOMPETENCE TO ABA-T IN LEWIS FEMALE RATS.

To analyze the ontogeny of DTH immuno-competence and to insure immuno-competent animals were used in this study, we wished to determine the optimum age for ABA-T priming. Animals of five different ages were immunized with 100mcg ABA-T/IFA or 100mcg ABA-T/CFA in the four footpads as shown in Figure 8. Twelve days later, the animals were skin tested with 100mcg ABA-6-OVAL/site. The results shown in Figure 8 clearly indicate that animals primed at 4 weeks of age are immuno-incompetent. Animals two weeks older

responded more strongly and by 8 weeks of age, all animals were immuno-competent and remaind so up to at least 18 weeks of age. We have previously reported that male Lewis rats require several more weeks to mature immunologically compared to females (Bullock, et al., 1980).

SENSITIZATION AND *IN VITRO* CHALLENGE OF RAT SPLEEN CELLS: PRIMING DOSE REQUIREMENTS

Several groups of Lewis female rats were primed with either 5, 50, or 500mcg ABA-T/IFA in each footpad. On days 10 or 20 following immunization, spleen cells were put into culture for 6 days with various antigen doses. Table I shows the maximum mean CPM and the maximum stimulation index. The test antigens used were either ABA-T or ABA-39-HGG and the dose ranges consisted of 5 fold dilutions from 625 to 1 mcg/ml. As can be seen, only cultures with ABA-T or ABA-39-HGG gave significant stimulation. The 5 and 500mcg priming doses were inferior compared to immunization with 50mcg as a priming dose. Both this and subsequent experiments suggested that 50-100 mcg ABA-T/IFA are required to produce srong *in vitro* responses. ABA-T/IFA was sufficient to prime animals for subsequent potent blastogenesis and, although rats primed with ABA-T/CFA responded *in vitro*, background levels were 2-4 times greater than those obtained with ABA-T/IFA primed animals (data not shown).

CULTURE AND CHALLENGE OF SPLEEN CELLS FROM RATS PRIMED VIA FOOTPAD INJECTION: EFFECTS OF TIME AFTER PRIMING AND FORM OF CHALLENGE ANTIGEN.

To determine the optimum time after priming via footpads for *in vitro* responses, rats were primed with 100mcg ABA-T/IFA in the footpads. Spleen cell cultures were then established 5, 10, 15, and 24 days after priming. Cells were stimulated *in vitro* with either ABA-T, ABA-3.6-HGG, ABA-6-Oval, or ABA-39-HGG, at 1-625mcg/ml in serial 5 fold dilutions. Table II presents the stimulation obtained with the optimal antigen concentration, as defined by that dose yielding maximum tritiated thymidine incorporation. Table II presents the average CPM +/- the SEM with the resulting stimulation indicies shown in parentheses. All antigen stimulated cultures produced stimulation indicies greater than 2.0 when cells were cultured 10 days post-priming. The small molecular weight original priming antigen ABA-T gave the lowest stimulation with a stimulation index (2.09); whereas, ABA-39-HGG induced the strongest blastogenic reaction with a stimulation index of 19.8 on day 10.

It should be noted that the stimulation indices are directly proportional to the valence of the antigen used and not the molecular weight, since the smaller ABA-6-Oval molecule yielded an

index 10 times that of the larger ABA-3.6-HGG. This also argues against a major enhancing effect due to the Fc component of the HGG carrier. Also, later experiments produced strong *in vitro* stimulation following challenge with either ABA-Insulin or ABA-Albumin (data not shown).

In summary, blastogenic responses of cells from animals primed with ABA-T/IFA in the four footpads were maximal when cells were harvested for culture on day 10. Spleen cells responded poorly to ABA-T challenge *in vitro*. When ABA-proteins were used as *in vitro* challenge antigens, responses were much stronger than those obtained with ABA-T and were largely dependent upon the valency of the antigen.

EFFECT OF IP ROUTE OF ANTIGEN ADMINISTRATION AND THE KINETICS OF *IN VITRO* RESPONSE WITH SPLEEN CELLS.

In a second series of experiments, the IP route of administration was studied. Adult animals were injected with 100mcg ABA-T/IFA IP and spleen cell cultures were established and challenged *in vitro* on days 5, 10, 15, 20, and 25 post-priming. Table III tabulates the results of these experiments. By day 15 post-priming, stimulation of the ABA-T primed cells by ABA-T challenge produced a 16-fold increase over background responses. Reactivity peaked on day 20 presenting a 42-fold increase. When cells were cultured 25 days

post-primary, the responses were dramatically depressed. The same pattern of response was obtained with ABA-39-HGG challenge, reaching a 50-fold increase over background on day 20 which diminished to near background levels 5 days later. Two additional challenging antigens were tested, although not over the entire time course. ABA-59-HGG initiated a strong response on day 15 while ABA-3.6-HGG, as seen in Table II, produced poor stimulation again.

Figure 9 graphically compares the effects of IP vs. FP priming. The panel on the left shows the response of animals when primed via the IP route and challenged in vitro with either ABA-T or ABA-39-HGG, while the panel on the right shows the response of animals primed via FP injection. First and foremost, for spleen cell blastogenesis, immunization via the IP route is enormously potent when compared with FP immunization. Second, ABA-T with a valency of one is as stimulatory in animals primed via the IP route as the multivalent molecule ABA-39-HGG. Strikingly, footpad injected animals showed essentially no response to the priming antigen ABA-T but were immune as indicated by the strong responses they were capable of mounting to ABA-39-HGG. In contrast to this data, the in vivo response to priming by either the intraperitoneal route or the four footpads produced equally strong skin reactions (cf. Figure 6).

In addition, the kinetics of immune response following IP or FP injection were different. Footpad immunized animals, although displaying less stimulation than their IP injected counterparts,

gave maximum responses at 10 days for on ABA-protein (ABA-HGG), and responded maximally to another ABA-protein on day 24 (ABA-6-Oval, cf. Table II.

EXAMINATION OF IMMUNE SPECIFITY IN THE IN VITRO BLASTOGENESIS ASSAY

To ensure that the responses detected were indeed immune specific, two groups of animals were studied in the blastogenesis assay. One group was immunized with ABA-T/IFA IP and spleen cells were harvested 20 days later and placed into culture. The other group consisted of animals that received no previous treatment but were cultered at the same period. As shown in Table IV, only immune animals responded to <u>in vitro</u> challenge with ABA-T or ABA-39-HGG. Neither the immune group or the non-immune group responded to challenge with the carrier protein HGG. This experiment provides evidence of the immune specificity of the <u>in vitro</u> blastogenic response.

VARIATION IN RESPONSE BETWEEN INDIVIDUALS OF THE SAME STRAIN.

Experiments to this point were carried out with cells pooled from several rat spleens. We wished to determine if this highly inbred strain continued to permit variation between individual animals.

One method to test for individual variation in animals treated in identical fashion, is to separately culture cells from individual spleens. All animals were housed and maintained similarly. Females of the same age +/- a few days were primed at the same time with the same emulsion of antigen and adjuvant. Several groups of animals were primed with ABA-T/IFA IP on the same day. At 16, 18, 20, or 23 days after priming, individual spleen cell suspensions were cultured with or without ABA-T. The results are shown in Figure 10. Strong positive responses were seen in approximately 3/4 of the animals with the greatest stimulation again seen on day 20 as in Figure 9. Low responses were detected in approximately 1 of 4 animals tested on each day. Clearly, individual variability must be a consideration even in these highly inbred animals. This is clearly in contrast to the more limited <u>in vivo</u> variation. For example, when 17 ABA-T immune rats were tested at one time in a particular experiment, we found that all 17 reactions on the right side of the animal to be 14-17mm in diameter and all 17 reactions on the left side of the animal to be 14-17mm in diameter.

DISCUSSION

Blastogenesis is frequently used as an *in vitro* correlate to *in situ* T-cell phenomena such as DTH skin reactivity. We here compare and contrast these two immune reations using immune rats which produce both strong, classic DTH skin reactions and large extensive antigen induced blastogenesis *in vitro*. The antigen chosen was the small, completely defined molecule ABA-T, which produces T-cell immunity in the absence of significant antibody (Becker, et al, 1975, Collotti and Leskowitz, 1970). We found that *in vivo* and *in vitro* responses differed as to 1) optimal site for sensitization, 2) kinetics of sensitization, 3) sensitivity to high dose suppression and 4) the uniformity of response between individual animals.

To optimize the ABA-T *in vivo* DTH model, we tested eight rat strains to select that strain most responsive to ABA-T. Seven strains reponded, with female Lewis rats producing the largest, most consistent reactions. Females were used because we previously found that male Lewis rats required several more weeks than females to immunologically mature (Bullock, et.al. 1980). ACI rats failed to respond to ABA-T priming even when ABA-T was given at several doses in a variety of adjuvants. Since ACI rats did respond to PPD when sensitized with ABA-T in mycobacterial adjuvant, this genetic non-responsiveness appears to be specific.

After testing a large range of ABA-T doses, we found that as

little as 50-100 mcg/animal induced sensitization. However, doses up to 2000 mcg/animal did not significantly suppress this maximum sensitivity 10-14 days post-priming. Higher doses did shorten the persistence of such sensitivity, possibly due to stimulaton of an active suppressor mechanism or accelerated exhaustion of a responding cell population. DTH skin reactivity peaked 10-14 days after priming and dropped dramatically, in contrast to the high level of persistant sensitivity observed with guinea pigs. Early appearance and loss of maximum DTH in rats has previously been reported. (Bullock, 1976, Bullock, 1978, Kruger, et al., 1971, Sobel, et al., 1975). Limited sensitivity does however persist up to 61 days after sensitization. (cf. Figure 6).

Although the traditional adjuvant and route of administration for induction of anti-ABA-T DTH have been CFA and footpad injection, to our surprise, ABA-T in incomplete Freund's adjuvant, given intraperitoneally, works equally well. Intraperitoneal administration is in fact more convenient, less crippling, and is essential to strong <u>in vitro</u> spleen cell responses to ABA-T antigen (cf. Figure 9). In contrast to the findings reported here, Lawn and Leskowitz (1980) reported that ABA-T/IFA, was not immunogenic, but in fact, inhibited subsequent immunization, similar to earlier ABA-T/IFA studies in our laboratory with guinea pigs (Bullock, et al., 1975). The most likely explanations for their negative results are the timing and the challenge antigen used. They appear to have tested for DTH to ABA-T/IFA on day 21. This was unfortunate since,

as we previously reported, days 10-14 are optimum for this short lived reactivity (20-Bullock <1978>). In addition, the dose of ABA-Insulin used was a weak challenging antigen as shown in Figure 4.

Conditions for in vitro reactivity to ABA-T differ considerably from those observed for DTH skin reactivity. A narrow senitization dose range exists, with both high, 500 mcg/animal, and low, 5 mcg/animal, doses of ABA-T producing poor priming for in vitro spleen cell responses. This is in contrast to the intense skin reactions obtained following priming with 50-200 mcg ABA-T/animal. Although high in vivo priming doses suppressed in vitro responses, high doses of either ABA-T or ABA-proteins were found to be optimal for in vitro induction of blastogenesis as reported earlier (Hanna, et al., 1973). Blastogenesis was not stimulated with a conjugate of low ABA/protein ratio (i.e. ABA-3.6-HGG). With appropriate antigen doses, DTH skin reactions were remarkably uniform with little individual variation, in contrast to in vitro responses where considerable variation existed among the responses of immune spleen cells from different individuals.

The kinetics of in vivo and in vitro reactivity also differ dramatically, with DTH skin reactivity peaking at 10-14 days (figure 6); whereas, spleen cells obtained 10-14 days post priming were only starting to respond in vitro. However by day 20, when skin reactions were weak, in vitro reactivity was maximum (Table 3). By day 23, in vitro spleen cell responses were mostly lost whereas,

some skin reactivity persisted up to 60 days. This rapid loss of *in vitro* spleen cell reactivity may reflect exhaustion of a population of cells or the appearance of splenic suppressor cells. Recent cells mixing experiments suggest that both activities occur (paper in preparation). Meyer-Bloch, et al., (1980) reported a similar discrepency between peak skin reactivity (day 14) and maximum lymphocyte reactivity *in vitro* (day 35) following sensitization of guinea pigs with antigen in IFA.

The ABA-T "family of molecules" have been used to study DTH in guinea pigs, rabbits, rhesus monkeys, and rats (Nauciel & Raynaud, 1971, Moon, et al., 1977, Mackler, et al., 1971, and Bullock, 1978). As already mentioned, considerable species variability exists in response to these antigens. Most of the early work with this antigen involved sensitization of guinea pigs with ABA-T in CFA adjuvant, since ABA-T in IFA adjuvant induces suppressor cells and causes tolerance in these animals (Bullock, et al., 1975). Perhaps antigen in IFA acts similarly in rats but suppressor cells evolve more slowly permitting a transient "window of response" 10-14 days post priming.

In vitro reactivity of ABA-T primed cells has received little attention, although ABA-T primed lymph node cells (LNC) have been studied. Becker, et al., (1973) obtained positive but weak guinea pig LNC stimulation 14 days post sensitization. Nauciel and Raynaud (1971) reported weak guinea pig stimulation indicies 16-100 days after priming. Hanna, et al., (1973) obtained both strong

blastogenesis and marked MIF production with LNC 21 days after priming guinea pigs with ABA-T similar to the 20 day maximum blastogenesis findings reported here for spleen cells. Bellone, et al.,(1975) also reported that maximum LNC stimulation occurred 3 weeks after antigen priming but reported poor correlations between blastogenesis and MIF production. Furthermore, they reported that the kinetics of immune activity of LNC differed from that of peritoneal exudate cells (PECS). However, only one previous report mentions use of spleen cells. Lawn and Leskowitz (1980) reported that spleen cells were generally nonstimulatable or even suppressive; neither data nor protocals were given. We report here that under appropriate conditions, spleen cell responses were comparable to those reported for both PECS and LNC.

From the data presented here and from the results of the investigators reported above, we conclude that organ "compartmentalization" of the immune system occurs. That is, specific immune response may be demonstratable in one organ but not another organ from the same animal at a given time. The major differences seen between animals primed via footpads versus intraperitoneal injection could be due to differing "traffic patterns" of various T-cell subpopulations. Conceivably, footpad injection allows for the development and preferential migration of T suppressor cells to the spleen. Intraperitoneal injection, however, may allow for the development of T effector cells that migrate rapidly to the spleen. Soon after, T suppressor cells migrate into

the spleen in sufficient numbers to inhibit spleen cell blastogenic response. This study demonstrated unique differences between two assay systems sometimes believed to measure the same immune cell population and identifies potential "pitfalls" to be considered in comparative analysis using two different assays.

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FIGURE 1. Relative DTH Responsiveness of Rat Strains to ABA-T Antigen

Eight different strains of rats were immunized with 50mcg ABA-T/IFA in the four footpads as described in the methods. Fourteen days after sensitization, all animals were challenged intradermally with 50mcg ABA-protein. Skin reaction diameters were measured 24 hours later. Shown are mean reactions (mm diameter) of 5-6 animals/group, +/- the SEM.

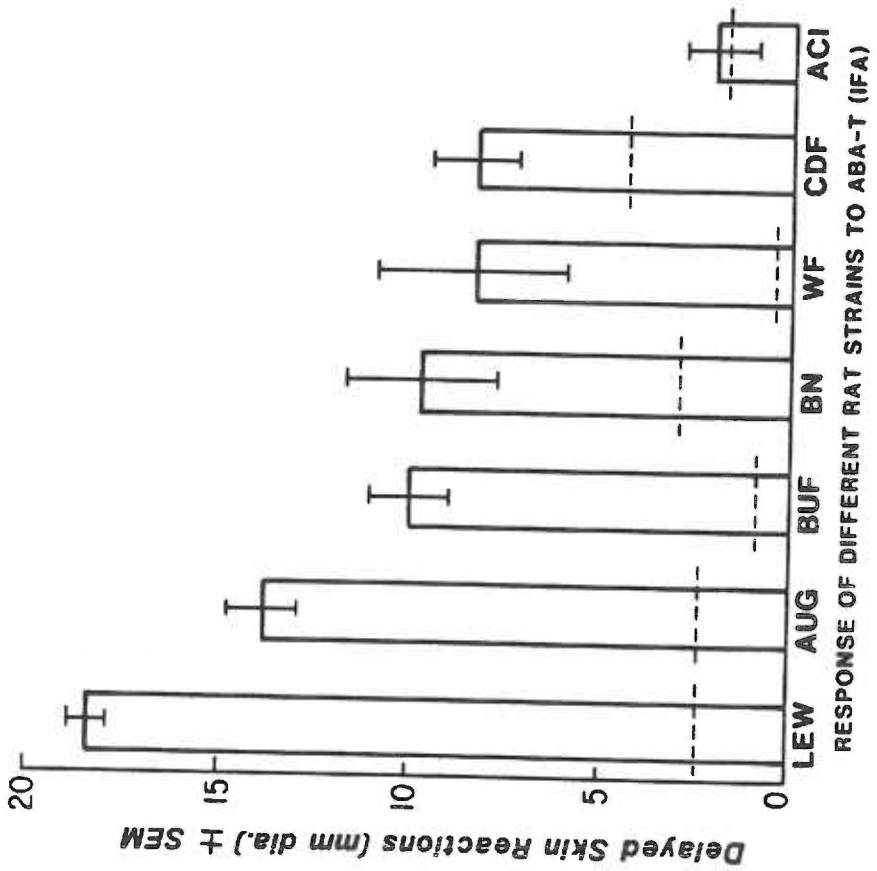

Delayed Skin Reactions (mm dia.) ± SEM
0
5
10
15
20
LEW
AUG
BUF
BN
WF
CDF
ACI
RESPONSE OF DIFFERENT RAT STRAINS TO ABA-T (IFA)

FIGURE 2. Effect of Antigen Priming Dose on Intensity and Persistence of Skin Reactions.

Five groups of 6 adult Lewis female rats per group received from 2-500 mcg/animal ABA-T/IFA in the four foot pads. An additional group received IFA only. Twelve days later, all animals were challenged with ABA-32-HGG intradermally. Skin reactions were measured 24 and 48 hours after challenge. The results displayed represents the average skin reactions (mm diameter), +/- the SEM.

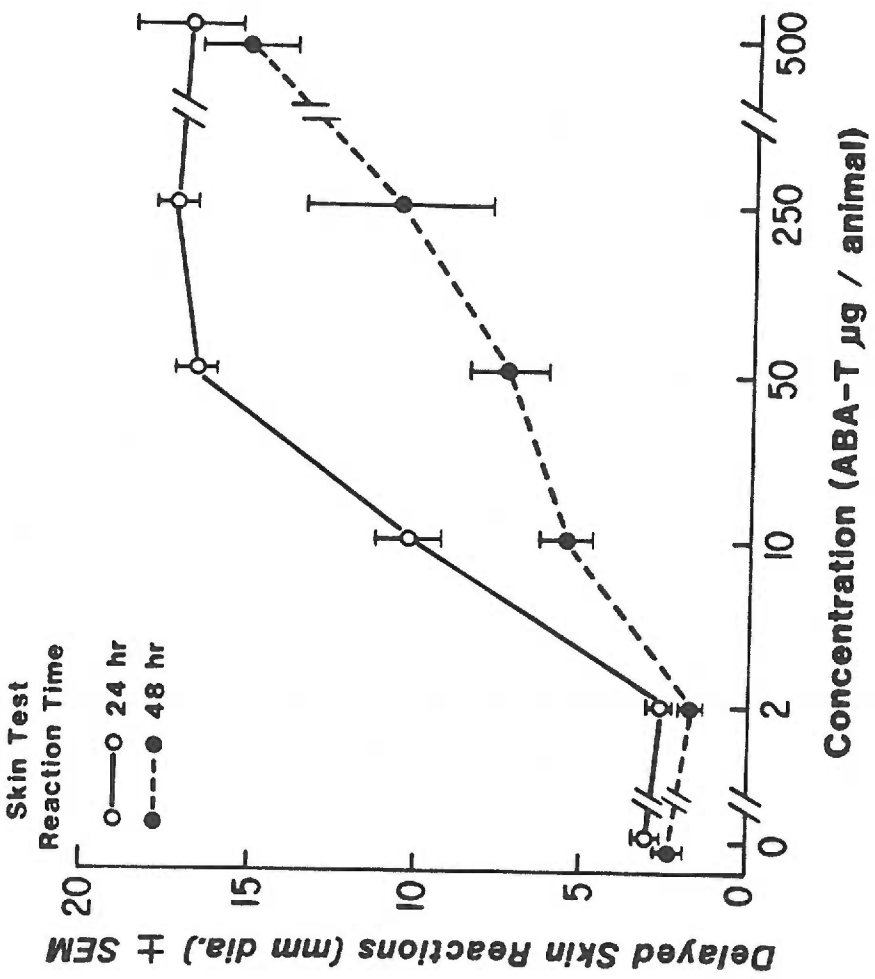
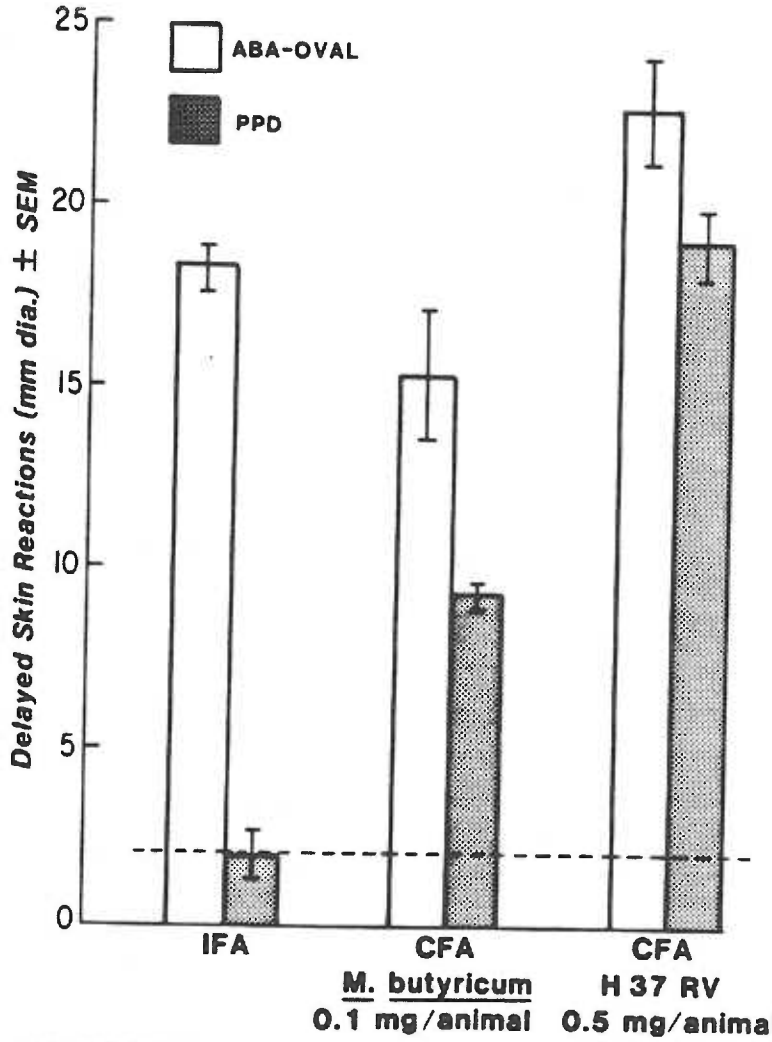

Delayed Skin Reactions (mm dia.) ± SEM
Concentration (ABA-T µg / animal)
Skin Test
Reaction Time
24 hr
48 hr
0
5
10
15
20
0
2
10
50
250
500

FIGURE 3. Role of Bacterial Adjuvants During Rat Sensitization to ABA-T.

Lewis rat were immunized with 50mcg ABA-T in IFA, IFA with 0.1 mg M. butericum/animal or IFA with M. tuberculosis (H37RV) 0.5 mg/animal. Two weeks later all animals were skin tested with 50mcg ABA-16-HGG per site. The data are presented as the average reaction diameters in millimeters of groups of 6 animals recorded 24 hours after skin testing, +/- the SEM. The dashed line represents the mean reaction diameters of non-sensitized rats.



ADJUVANT USED WITH ABA-T FOR IMMUNIZATION OF LEWIS RATS

FIGURE 4. Absence of Protein Specificity and Role of Challenge Dose in Eliciting a Response with ABA-protein.

Adult Lewis female rats were injected with 100mcg ABA-T/IFA intraperitoneally. Fourteen days later all animals, including a group of rats that had received no prior treatment (bars on left side of each group), were challenged intradermally with either 100mcg ABA-39-HGG, 100mcg ABA-28-HGG, 100mcg ABA-4-Insulin, or 20mcg ABA-4-Insulin. The next day skin reactions were measured. Results displayed are the average skin reactions (mm diameter), +/- the SEM. The dashed line represents the mean reacton diameters of non-sensitized rats.

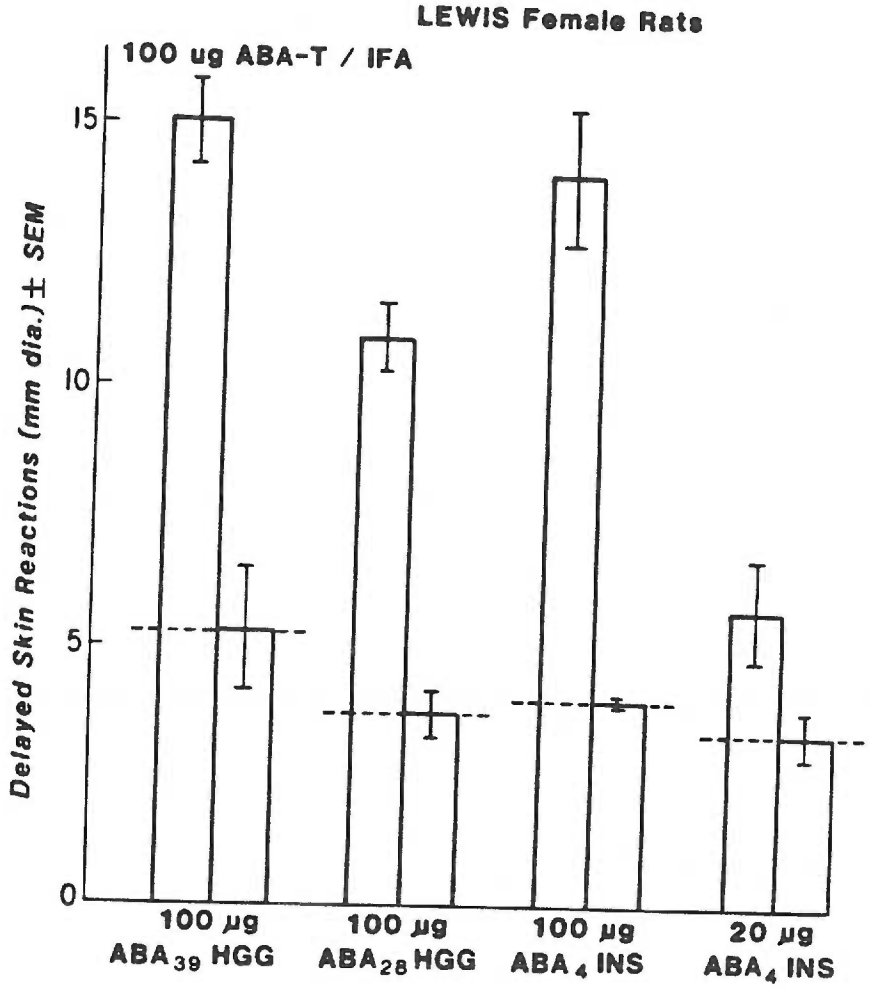

LEWIS Female Rats
100 ug ABA-T / IFA
Delayed Skin Reactions (mm dia.) ± SEM
15
10
5
0
100 µg ABA39 HGG
100 µg ABA28 HGG
100 µg ABA4 INS
20 µg ABA4 INS

FIGURE 5. Effects of antigen dose and time after sensitization on the DTH Response.

Delayed skin reactivity of animals primed with various doses of ABA-T. Adult female Lewis rats were immunized in the four foot pads with IFA only or 2, 10, 50, 250, or 500mcg ABA-T/IFA. Seven, ten and twenty-one days after priming, all groups were skin tested with 50mcg ABA-16-HGG/site. Skin reactions were read 24 hours later. Each point is the mean diameter (mm), +/- the SEM.

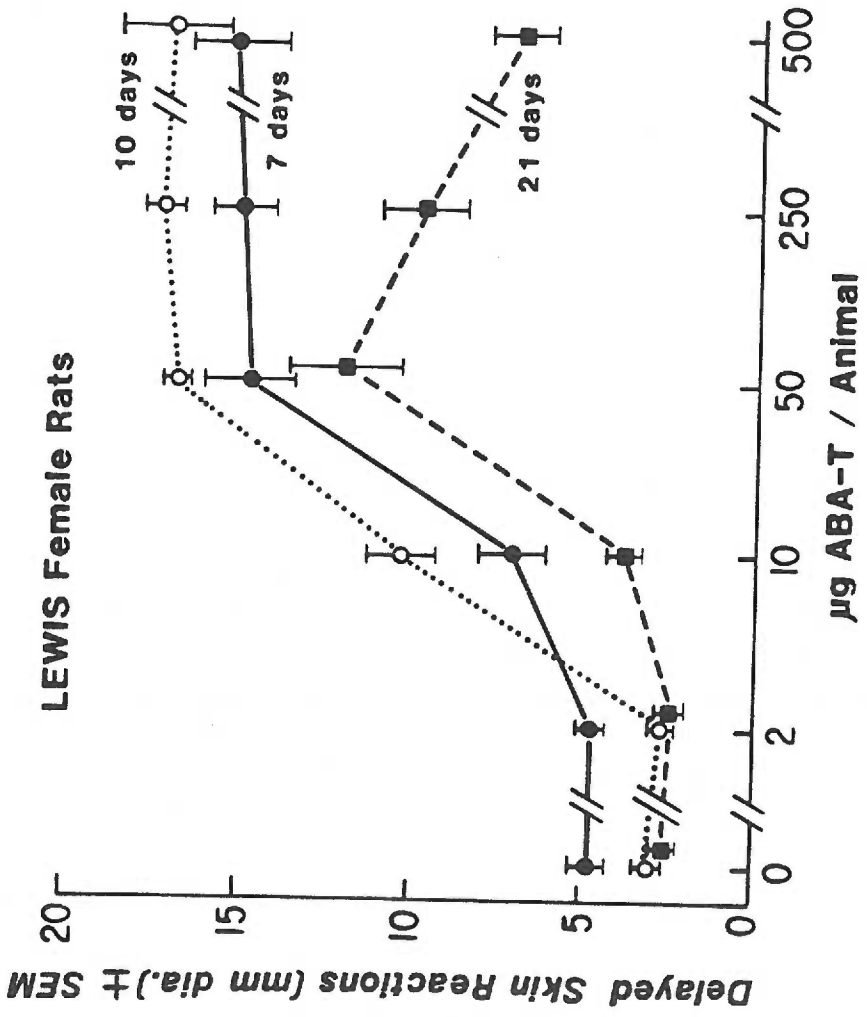

LEWIS Female Rats
Delayed Skin Reactions (mm dia.) ± SEM
20
15
10
5
0
0
2
10
50
250
500
µg ABA-T / Animal
10 days
7 days
21 days

FIGURE 6. Persistance of Anti-ABA-T Sensitivity.

Delayed skin reactivity to intradermal challenge various days after priming. Adult female Lewis rats were primed with 50mcg ABA-T/IFA given into the four foot pads. Different groups of animals were skin challenged on various days after immunization with 50mcg ABA-16-HGG/site. Twenty-four hours later, skin reactions were read. Each point is the mean diameter (mm), +/- the SEM.

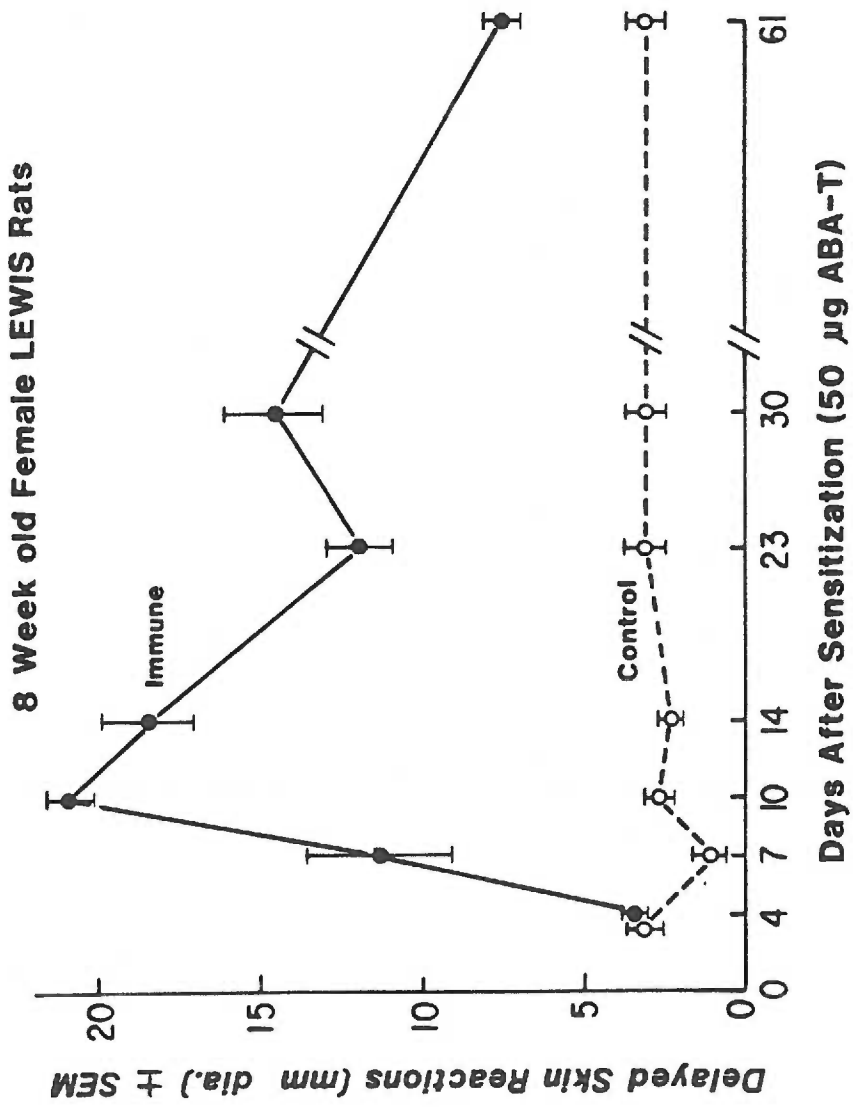

8 Week old Female LEWIS Rats
Immune
Control
Delayed Skin Reactions (mm dia.) ± SEM
Days After Sensitization (50 µg ABA-T)
0
5
10
15
20
0
4
7
10
14
23
30
61

FIGURE 7. Comparative Sensitizaton; Intraperitoneal vs. Footpad antigen Administration.

Adult female Lewis rats delayed skin responses obtained 10 days after priming. Animals were immunized to different doses of ABA-T/IFA either in the four footpads or intraperitoneally. ABA-11-Oval (50mcg/site) was given as skin challenge 10 days post-priming. Skin reactions were read 24 hours later. Each point is the mean diameter (mm), +/- the SEM.

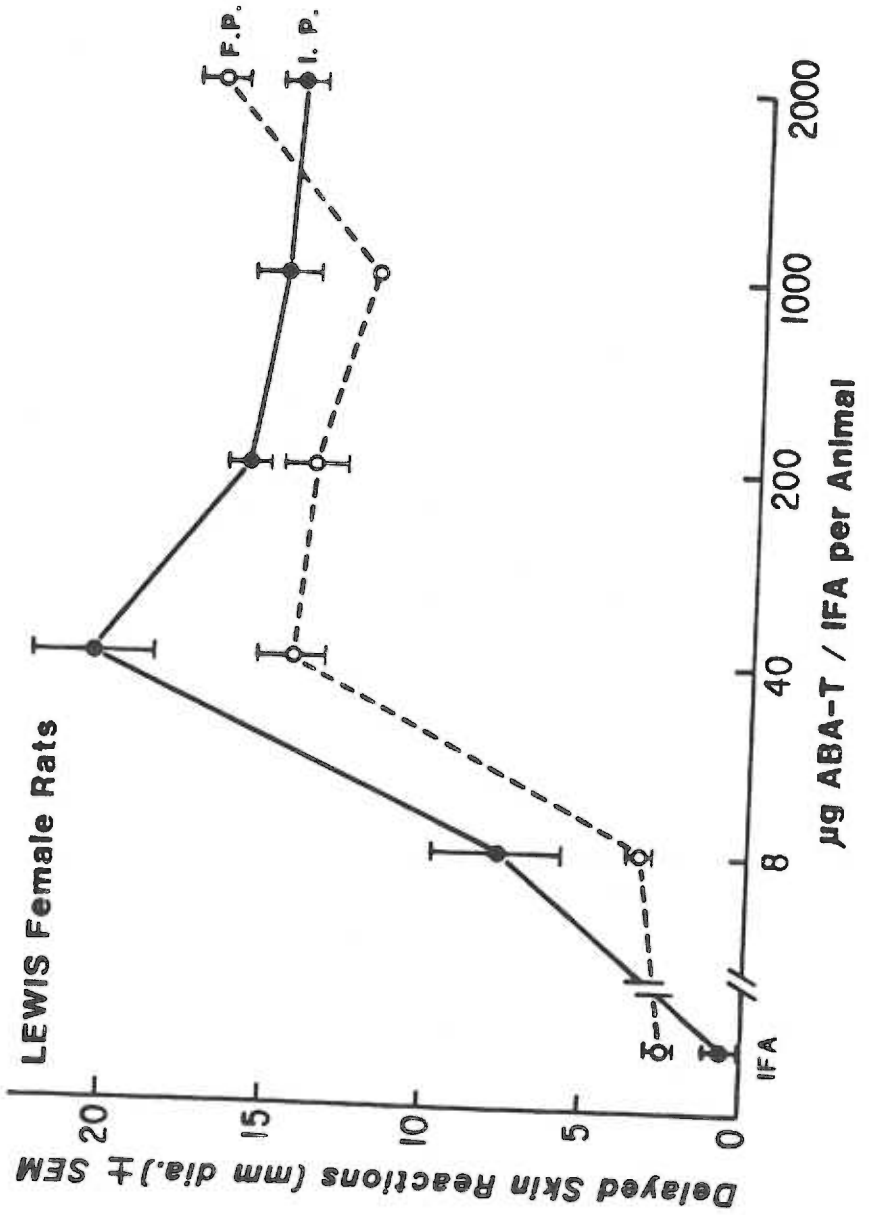

LEWIS Female Rats
Delayed Skin Reactions (mm dia.) ± SEM
0
5
10
15
20
IFA
8
40
200
1000
2000
µg ABA-T / IFA per Animal
F.P.
I. P.

FIGURE 8. Ontogeny of Immuno-competence to ABA-T in Lewis Rats.

Ontogeny of Lewis female rat delayed skin reactions. Various groups of rats at differnt ages (in weeks) were immunized with 100mcg ABA-T/IFA or 100mcg ABA-T/CFA given into the four foot pads. Twelve days after sensitization, animals were skin tested with either 100mcg ABA-6-Oval or 100mcg ABA-39-HGG per site. Twenty-four hours later, skin reactions were measured. Each point is the mean diameter (mm), +/- the SEM. The dashed line represents the maximum response obtained to skin challenge with ABA-protein in non-sensitized, 18 week old rats.

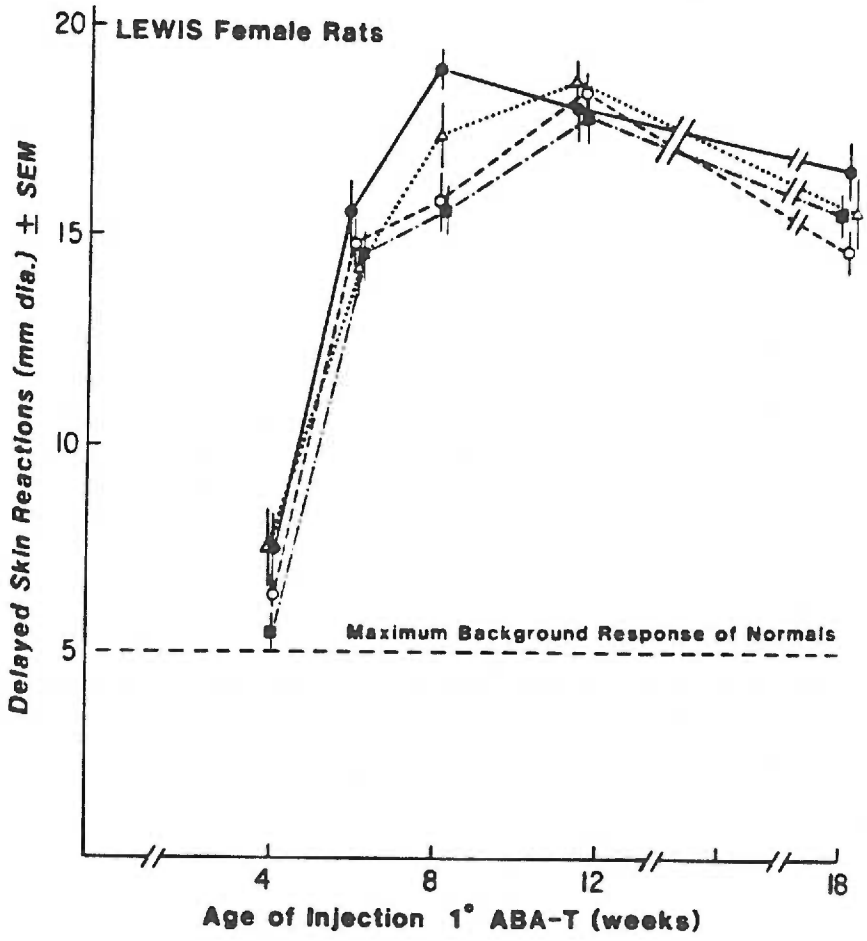

LEWIS Female Rats
Delayed Skin Reactions (mm dia.) ± SEM
20
15
10
5
Maximum Background Response of Normals
4
8
12
18
Age of Injection 1° ABA-T (weeks)

TABLE 1

In vitro secondary challenge of ABA-T primed adult Lewis female rat spleen cells to various ABA-conjugated carriers. Animals were primed by footpad injection with either 5mcg, 50mcg, or 500mcg ABA-T/IFA. Spleen cells were cultured 10 or 20 days after priming with various ABA-conjugated antigens for 6 days. One day prior to harvesting the cells, 0.5 mcCi ^{3}H-thymidine was added to each well. Values shown are the average maximum CPM yielded, +/- the SEM.

TABLE I

EFFECT OF DOSE AND TIMING ON IN VITRO RESPONSE

1° Dose ABA-T/IFA (F.P.) per animal		5 µg		50 µg		500 µg	
Culture initiation in Days Post-1°		10	20	10	20	10	20
	Nothing	1725 +/- 97	803 +/- 25	1874 +/- 190	1178 +/- 63	2542 +/- 190	1258 +/- 150
2° Antigen	ABA-T	2489 +/- 119	970 +/- 119	2875 +/- 492	7280 +/- 1050	2456 +/- 160	1516 +/- 90
		(1.44)	(1.21)	(1.53)	(6.17)	(0.96)	(1.21)
	$ABA_{39}HGG$	7500 +/- 1378	5349 +/- 1387	5690 +/- 836	15433 +/- 1548	6975 +/- 1002	4874 +/- 697
		(4.35)	(6.66)	(3.03)	(13.11)	(2.74)	(3.87)

TABLE II.

The same as Table I except all animals were primed with 100mcg ABA-T/IFA in the footpads and *in vitro* cultures were initiated at days 5, 10, 15, or 25 post priming.

TABLE II

MAXIMUM RESPONSE KINETICS OF LEWIS FEMALE RATS SENSTIZED TO ABA-T/IFA IN THE FOUR FOOT PADS

Culture initiation in days since sensitization	5	10	15	24
Antigen in culture				
Nothing	798 ± 52	1070 ± 168	1886 ± 159	1383 ± 28
ABA-T	1179 ± 358	2240 ± 383	1471 ± 142	1196 ± 169
	(1.48)	(2.09)	(0.78)	(0.87)
$ABA_{3.6}HGG$	1505 ± 773	2476 ± 212	1892 ± 300	1598 ± 122
	(1.89)	(2.31)	(1.00)	(1.16)
$ABA_{39}HGG$	1066 ± 309	21183 ± 162	17255 ± 1847	4366 ± 2540
	(1.34)	(19.80)	(9.15)	(3.16)
$ABA_{6}Oval$	1022 ± 79	5100 ± 482	1909 ± 289	17938 ± 2576
	(1.28)	(4.77)	(1.01)	(12.97)

TABLE III.

The same as Table I except all animals were primed with 100mcg ABA-T/IFA intraperitoneally and *in vitro* cultures were initiated at days 5, 10, 15, or 25 post priming.

TABLE III

MAXIMUM RESPONSE KINETICS OF LEWIS FEMALE RATS SENSITIZED TO ABA-T/IFA INTRAPERITONEALLY

Culture initiation in days since sensitization	5	10	15	20	25
Antigen in culture					
Nothing	1972 ± 49	360 ± 19	725 ± 52	1332 ± 99	2464 ± 262
ABA-T	2487 ± 252 (1.26)	712 ± 686 (1.98)	11630 ± 611 (16.04)	56360 ± 2598 (42.31)	5277 ± 484 (2.14)
$ABA_{3.6}HGG$	2589 ± 140 (1.31)	426 ± 11 (1.18)	1762 ± 470 (2.43)	Not Done	Not Done
$ABA_{39}HGG$	2323 ± 163 (1.18)	444 ± 20 (1.23)	21135 ± 1655 (29.15)	66579 ± 2828 (49.98)	4906 ± 1927 (1.99)
$ABA_{59}HGG$	2266 ± 224 (1.15)	474 ± 20 (1.32)	12116 ± 1049 (16.71)	Not Done	Not Done

TABLE IV.

Lewis female rats were either untreated before culture or were immunized with 100mcg ABA-T/IFA I.P. Twenty days later spleen cell cultures were established with or without antigen or mitogen. Five days after initiation of the culture, 0.5mcCi/wellof tritiated thymidine was added and all cultures were harvested one day later. The maximum obtained mean CPM, +/- SEM are displayed with the stimulation index immediately shown below in parantheses.

TABLE IV

MAXIMUM RESPONSE KINETICS OF LEWIS FEMALE RATS SENSITIZED OR NOT SENSITIZED WITH ABA-T/IFA INTRAPERITONEALLY

Challenging antigen in culture	No Treatment (Non-Immune)	100 mcg ABA-T/IFA I.P. 20 days before culture
Nothing	847 ± 80	469 ± 88
Concanavalin A	58583 ± 2573 (69.2)	58324 ± 5408 (124.1)
ABA-T	539 ± 149 (0.6)	4847 ± 893 (10.3)
ABA_{39}^{HGG}	755 ± 251 (0.9)	16165 ± 3190 (34.4)
HGG	881 ± 82 (1.0)	767 ± 90 (1.6)

FIGURE 9. Significant In Vitro Response Requires Intraperitoneal Sensitization.

Maximum in vitro blastogenic reponse to ABA-T or ABA-HGG challenge. Adult Lewis female rats were immunized with 100mcg ABA-T/IFA either in the footpads (right panel) or intraperitoneally (left panel). At days 10, 15, 20, or 25 post priming, spllen cells were cultured with antigen for 6 days. One day prior to harvesting, 0.5 mcCi ^{3}H-Thymidine was added to each well.

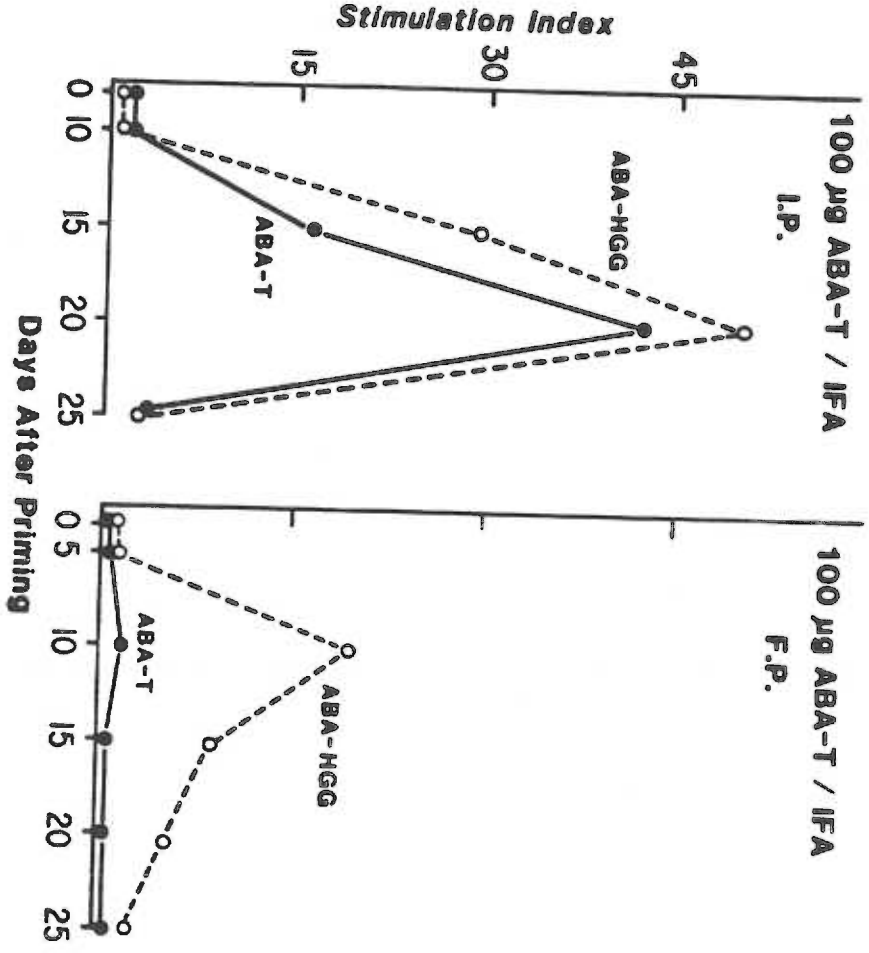
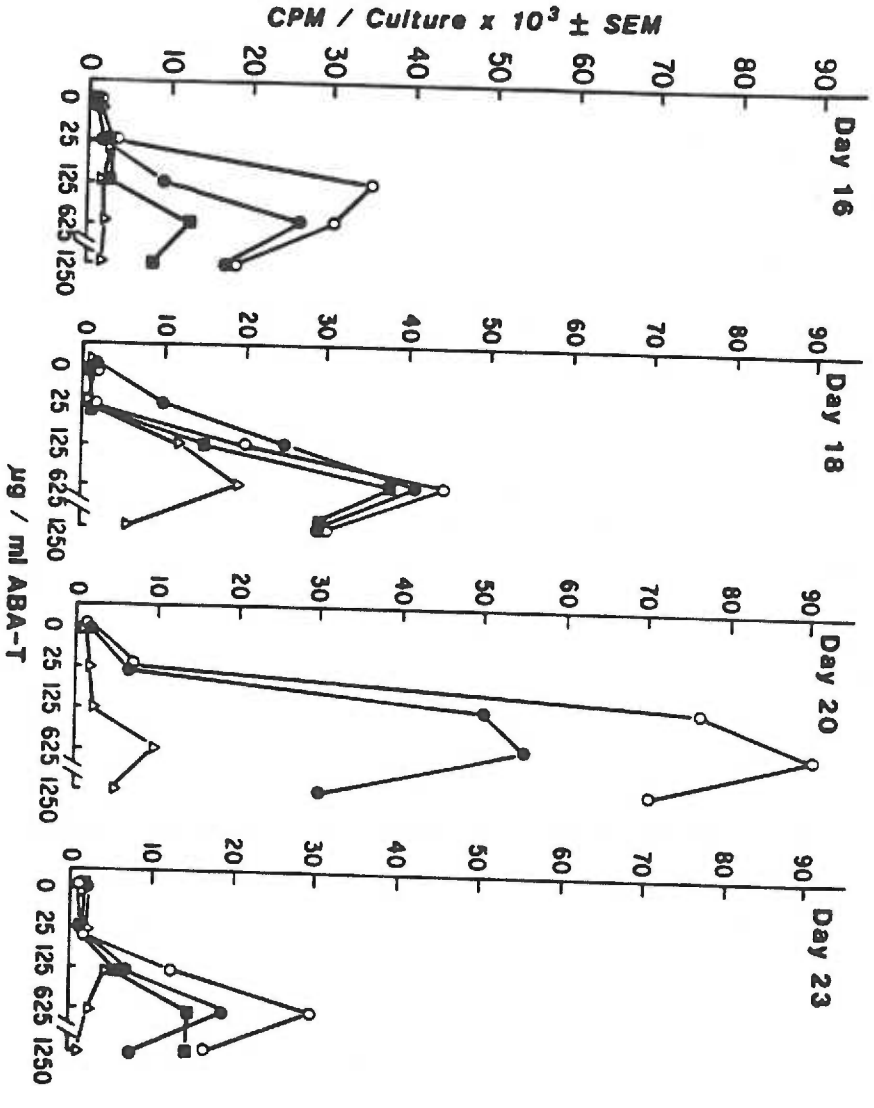

Stimulation Index
15
30
45
0 10 15 20 25
Days After Priming
100 µg ABA-T / IFA
I.P.
ABA-T
ABA-HGG
100 µg ABA-T / IFA
F.P.
0 5 10 15 20 25
ABA-T
ABA-HGG

FIGURE 10. Large Variation in Response to In Vitro Challenge Between Individuals of the Same Strain.

In vitro blastogenic response of adult Lewis female rats immunized to ABA-T/IFA. Animals received 100mcg ABA-T/IFA intraperitoneally. Sixteen, 18, 20, or 23 days later individual animal spleen cell cultures were established with or without ABA-T. Cultures went for 6 days and one day prior to harvesting, 0.5 mcCi 3-H Thymidine was added to each well. Each point is the mean diameter (mm), +/- the SEM.


CPM / Culture x 10³ ± SEM
Day 16
Day 18
Day 20
Day 23
0 10 20 30 40 50 60 70 80 90
0 25 125 625 1250
µg / ml ABA-T

SENSITIZED TO ABA-T. II. DO SENSITIZATION AND TOLERANCE DISPLAY SIMILAR SPECIFICITIES?

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ABSTRACT

We have prepared two antigens, azobenzenearsonate (ABA) -N-chloroacetyl -L-tyrosine (ABA-T) and ABA -N-cloroacetyl -L-tryptophan (ABA-try). These immunogens have been compared as to immunogenicity and cross-reactivity *in vivo* and *in vitro* and compared as to tolerogenicity or suppressor activity *in vivo* and *in vitro*. We learned that positive immune activity, both DTH skin reactions and *in vitro* T-lymphocyte transformation, are ABA and amino acid specific, i.e. little cross-reactivity was observed between ABA-T and ABA-try. In contrast, both *in vivo* tolerance and *in vitro* suppression were completely cross-reactive, preventing reactions regardless of the amino acid component used. The evidence presented here suggests that the fine specificity of DTH and tolerance to ABA-T and ABA-try differ.

INTRODUCTION

Various structural analogues of azobenzenearsonate- N-chloroacetyl- L- tyrosine (ABA-T) have been used extensively because of their known structure and the capacity of these antigens to induce DTH without inducing significant antibody, thus permitting the investigator to study cell mediate immunity without interference from antibody regulatory effects. We previously reported that juvenile tolerance to ABA-T in rats can be broken with ABA-protein (Bullock, 1979). In this paper we ask the question: was tolerance broken by recruiting new HGG carrier specific helper cells or by sensitizing animals with ABA coupled to a different amino acid, i.e. a new antigen. To answer that question, we have prepared two antigens, ABA-T and ABA-try. These immunogens have been a) compared as to immunogenicity and cross-reactivity _in vivo_ and _in vitro_ and b) compared as to tolerogenicity or suppressor activity _in vivo_ and _in vitro_. We learned that positive immune activity, both DTH skin reactions and _in vitro_ blastogenesis, are ABA- and amino acid-specific, i.e. little cross-reactivity was observed. In contrast, induction of _in vivo_ tolerance and _in vitro_ suppression of lymphocyte blastogenesis were completely cross-reactive regardless of the amino acid component used. This suggests that ABA-HGG or catabolic products do not break tolerance by providing new ABA-

amino acid antigens.

Materials and Methods

Rats: Lewis female rats were purchased from either Jackson Laboratories, Bar Harbor, Maine, or Microbiological Assoc., Inc., Walkersville, Maryland. In some experiments, Lewis female rats were from our breeding stocks purchased from the above sources.

Chemicals: p-Arsanilic acid was purchased from Eastman Kodac Co., Rochester, New York. N-chloroacetyl-L-tyrosine and human gamma globulin (HGG), Cohn Fraction II used to prepare ABA-59-HGG and ABA-3.6-HGG were purchased from Sigma Chemical Co., St. Louis, Missouri. N- chloroacetyl- L- tryptophan was purchased from Chemalog Corp., South Plainfield, New Jersey. HGG used in preparing ABA-39-HGG was purchased from Pentex Inc., Kankakee, Illinois. Hen egg ovalbumin (Oval) used to prepare ABA-6-OVAL was purchased from Pentex Inc., Kankakee, Illinois.

Preparation of antigens: Preparation of ABA-T and ABA-proteins has been described previously (Bullock, 1978). Essentially, the procedure is a modified version described by Tabachnick and Sobotka (1959). Briefly, the diazonium salt of p-arsanilic acid was

conjugated to the chloroacetylated amino acid tyrosine or tryptophan to form p- azobenzenearsonate- N- chloroacetyl- L- tyrosine (ABA-T) or p- azobenzenearsonate- N- chloracetyl- L- tryptophan (ABA-try). ABA-proteins were produced in a similar manner and dialyzed extensively against normal saline. ABA-T and ABA-try, due to their small molecular weights, could not be dialyzed and instead, were acid precipitated and washed three or four times. ABA-T, ABA-try, and the ABA-proteins were resuspended in Sorensen's phosphate buffer saline (PBS) and the pH established to 7.2-7.4 with 1N NaOH. Protein concentrations were determined by the Lowry method (1951) and the degree of ABA substitution on the proteins or the concentration of ABA-T and ABA-try were determined spectrophotometrically. The ABA component on ABA-proteins and ABA-T were determined assuming an extinction coefficient of :

Σ 1M = 10,500 at 490nm in 0.1N NaOH.

The extinction coefficient for ABA-try was determined to be:

Σ 1M = 5,001 at 396nm in 0.1N NaOH.

Immunization: All rats received 0.1 - 0.5ml Equi-Thesian (prepared in our laboratory) intraperitoneally (IP) as anesthesia prior to all procedures. Antigens for injection were prepared by emulsifying equal volumes of antigen in PBS in either incomplete

Freund's adjuvant (IFA, Difco Labs, Inc.) or complete Freund's adjuvant containing M. tuberculosis H37RV (CFA, also Difco Labs., Inc.). Animals were given 0.2ml of the antigen emulsion either IP or equally distributed into the four footpads (FP).

Induction of DTH: Animals were anaesthetized and their sides shaved. Intradermal injections (ID) of 0.1ml volumes containing the test antigen dissolved in PBS were administered up to 3 sites per side since multiple injection sites were found not to significantly affect reaction diameters. The diameter of induration and erythema was measured (mm) after at least 24 hours. When both induration and erythema were present, their reaction diameters were found to be comparable. If only induration or erthema was present, then this measurement was recorded as the reaction diameter. Arthus-like reactons were not observed.

In vitro spleen cell culture: Animals were anesthetized and exsanguinated prior to removal of spleens. The animals were sacrificed by cervical separation and the spleens were removed. Spleen cells were teased out into ice cold Eagle's Minimal Essential Medium, with Earle's salts and Hepes buffer (MEM, Flow Labs., Inc., Inglewood, CA). Cells were washed 3 times in cold MEM and then counted with a hemocytometer. The complete culture medium

contained, in addition to MEM, 10 mM nonessential amino acids, 100mM Na Pyruvate, 200 mM L-Glutamine, 2.0 mg/ml Na bicarbonate, 100 units/ml penicillin, 100 mcg/ml streptomycin, 2.0×10^{-5} ^{2}mercaptoethanol and 5% heat-inactivated fetal calf serum (FCS, Flow Labs., Lot # 4056012). The complete culture medium was typically made in 100ml batches or multiples thereof on the day of use. Cells were cultured at 2.0×10^{6} cells/ml in a final volume of 0.25 ml per well in Falcon Micro-Test II plates maintained in 5% CO^{2} and 95% air atmosphere at 37^{o}C. Cells and antigens or mitogen (Con A) were all diluted in complete culture medium prior to addition to culture wells. All experiments included Con A stimulated positive controls. In all experiments, Con A at its optimal concentration of 5 mcg/ml, elicited a response of >= 1.0×10^{5} counts per minute (CPM) of incorporated ^{3}H- Thymidine. To assess stimulation, 0.5mcCi ^{3}H-Thymidne (specific activity 20-25 Ci/mM, NEN) was added to each culture one day prior to harvesting. All cultures were maintained for six days. Cells were harvested onto glass fiber filters, dried and counted in Insta-gel (Packard Scientific) or a Omnifluor (NEN) - toluene (Baker) scintillation cocktail using a Searle Scintillation counter. Vials and pads without cells in scintillation cocktails gave an average of 50 CPM as background and were not subtracted out for calculations.

Statistics: For the <u>in vivo</u> DTH assay, each group contained

5-7 animals. The *in vitro* blastogenesis assay used 6 cultures of cells, pooled from 2-4 animals except where indicated, per antigenic or mitogenic dose. Usually 12, though sometimes six unstimulated cultures were used for negative controls. Means and the standard error of the mean (SEM) were calculated arithmetically. Statistical significance was calculated by using the Mann-Whitney U test. Probabilities of 0.05 or less ($p <= 0.05$) were considered statistically significant.

RESULTS

Elicitation of skin reactions from ABA-T primed animals usually employs challenge with various ABA-protein conjugates due to the rapid rate at which ABA-T diffuses from the reaction site (Nauciel & Raynaud, 1971). We first wanted to learn if, by increasing the challenge dose, we could study amino acid requirements for the elicitation of DTH skin reactions using ABA- amino acid as the challenging antigen.

DTH SKIN REACTIONS TO ABA- AMINO ACID CONJUGATES ARE SPECIFIC FOR BOTH THE ABA AND THE AMINO ACID COMPONENT.

Six adult Lewis female rats/group were immunized with CFA only or 100mcg/animal of either ABA-T/CFA or ABA-try/CFA. Twelve days later animals were skin tested with 100mcg PPD, 500mcg ABA-T, and 500mcg ABA-try. The resulting DTH skin reactions are shown in Figure 1. It can be seen that the reactions are amino acid specific, i.e. ABA-T primed animals failed to react to ABA-try; whereas, ABA-try primed animals failed to react to ABA-T. All animals responded to PPD as expected with CFA priming. Both ABA primed groups responded to ABA-protein presumably due to ABA conjugated to tyrosine and tryptophan residues within the HGG protein molecule. CFA-only-primed animals failed to respond to any

ABA-conjugates indicating the requirement for ABA priming. We also found that as previously shown (Nauciel and Raynaud, 1971) lower doses of ABA-T produced poor skin reactions (data not shown).

IN VITRO BLASTOGENESIS REACTIONS TO ABA IMMUNE CELLS RESPOND MAXIMALLY TO CHALLENGE WITH ABA-COUPLED TO THE SAME AMINO ACID.

We next wanted to learn if the observed specificity of skin reactions for a particular ABA- amino acid conjugate applied to stimulation of immune cell division in vitro. We therefore immunized adult Lewis female rats with 100mcg/animal ABA-try/IFA. IFA and intraperitoneal injections were used because we have previously shown that these conditions are critical to obtaining strong anti-ABA- amino acid reactions in vitro with rat spleen cells (Fennell and Bullock, 1983). Twenty days following immunizaton, spleen cells from these animals were pooled, cultured, and challenged with 1-1250 mcg/ml of either ABA-T or ABA-try or 1-625 mcg/ml of ABA-39-HGG. The resulting stimulation obtained is shown in Figure 2. Although ABA-T and ABA-try did cross-react, ABA-try was 3x more effective than ABA-T in stimulating ABA-try primed cells. The response was dose dependent with 5x more ABA-39-HGG and 25x more ABA-T being needed for maximum stimulation. Very high amounts of both ABA-T and ABA-try suppressed the response.

A similar experiment to that described above is presented in Figure 3. ABA-T primed animals are shown to respond to ABA-T but not to ABA-try. The overall results are similar to those in Figure

2 except that ABA-T primed animals failed to respond to ABA-try indicating a "one way" cross reactivity between these two molecules.

ABA-T GIVEN TO IMMATURE LEWIS RATS CAUSES SPECIFIC TOLERANCE.

Since both in vivo and in vitro responses to ABA-T or ABA-try require challenge with the matching amino acid, we wanted to ask if tolerance or specific immunosupression had a similar amino acid restriction. We therefore developed a method which permits induction of specific, long term tolerance to ABA-T. Three week old female Lewis rats were given IFA only or were tolerized with 0.32-1000 mcg/animal ABA-T/IFA in the four footpads. Three weeks later, all animals were primed with 50mcg ABA-T/animal in IFA and 50mcg HGG/animal in IFA at separate sites. Twelve days later, sensitized animals were skin tested with ABA-6-Ovalbumin and HGG, 50mcg each, at different sites. Test results are presented in Figure 4. As shown, the small monovalent antigen causes partial tolerance with as little as 0.32 mcg/ml ABA-T/animal; whereas, as much as 1000mcg ABA-T/animal completely fails to suppress the response to HGG. However, it was not clear whether tolerance was directed towards ABA alone or ABA-T, i.e. "is the amino acid an essential component of tolerance"?

JUVENILE TOLERANCE TO ABA-T AND ABA-TRY IS CROSS REACTIVE, EACH COMPLETELY BLOCKING SENSITIZATION TO THE OTHER ABA- CONJUGATE.

To test whether ABA tolerance blocks sensitization to all ABA-

amino acid conjugate or only to the original tolerizing amino acid conjugate, we tolerized with one ABA- amino acid antigen and then tried to sensitized the same animals, after they were immunologically mature, with the other amino acid conjugate. Four week old juvenile rats, 6 animals/group, were tolerized with ABA-T or ABA-try 50 mcg/animal. Control rats received IFA only. Animals were immunized 4 weeks later with IFA only or 50 mcg/animal of either ABA-T/IFA or ABA-try/IFA. Twelve days later, animals were skin tested with ABA-6-Ovalbumin and the results are shown in Figure 5. Both ABA-T and ABA-try induced tolerance which was completely cross-reactive in contrast to the amino acid specificity of both *in vivo* and *in vitro* recall reactions. Pretreatment with IFA alone as before did not preclude sensitization. This ABA specific tolerance could have resulted from clonal deletion of ABA recognizing cells regardless of amino acid recognition, potential activation of ABA specific suppressor cell clones induced by tolerance, or both.

ABA-ACTIVATED SUPPRESSION OF BACKGROUND THYMIDINE INCORPORATION.

We first suspected suppressor cell activity, by analyzing the findings that a) footpad priming does not prime for *in vitro* responses to either ABA-T or ABA-try and, b) CFA/antigen injections produce high levels of background thymidine incorporaton. When adult Lewis female rats were injected in the footpads with 100mcg ABA-T/CFA and spleen cells removed and cultured 10 days later, we observed antigen-induced suppression. Cultures were untreated or

received 0.2-125 mcg/ml of ABA-T, ABA-try, or 0.2-25 mcg/ml PPD. As shown in Figure 6, as little as 1-5 mcg/ml of all 3 "immunogens" supressed background thymidine incorporation. The presence of antigen specific splenic suppression cells could well explain the inability of such spleens to give positive responses *in vitro*. These results however do not suggest whether such *in vitro* suppression is specific for both ABA and the amino acid conjugate or only ABA as found following tolerance induction.

ABA-T PRIMED SPLEEN RESPONSE TO ABA-39-HGG IS BLOCKED BY EITHER ABA-T OR ABA-try IN THE ABSENCE OF CELLS RESPONSIVE TO EITHER ABA-T OR ABA-try.

To determine the specificity of *in vitro* suppression, we again used the finding that spleen cells from footpad primed ABA-T immune cells fail to respond to the ABA- amino acid conjugates. Adult Lewis rats were injected with 100mcg ABA-T/IFA in the 4 footpads. Twelve days later spleen cells were prepared and cultured with ABA-39-HGG stimulant +/- various doses of ABA-T or ABA-try (Figure 7). Although the cells did fail to respond to either ABA-T or ABA-try alone as previously shown (Fennell & Bullock, 1983), strong responses were elicited with ABA-39-HGG. As shown, both ABA-T and ABA-try were equally suppressive of this response with as little as 25 mcg/ml causing significant suppression. Although not shown, 125 mcg/ml of either of the reagents completely failed to suppress Con A activiation arguing against potential long term toxic effects

throughout the 6 day culture period. In addition, it should be emphasized that 25-625 mcg/ml of ABA-T and ABA-try produces maximum stimulation following peritoneal priming (cf. Figure 2 and 3). Thus, either ABA- amino acid conjugate alone activated suppressor cells, as suggested in Figure 6, or ABA- conjugates can block responding cell receptors regardless of the amino acid restrictions.

DISCUSSION

Much evidence suggests that DTH reactions can be induced only with immunogens possessing a protein component (Vassalli and McCluskey, 1971). By analogy, the tyrosine derivative of ABA-T would appear to present the smallest possible "protein", one amino acid! Several lines of evidence suggest that it is indeed the tyrosine end of this molecule which displays carrier function. Modification of the tyrosine structure, such as removal of the carboxyl or amino group, or acetylation of the amino group, affect the immunogenicity (carrier function) of the molecule with little effect on DTH specificity (Alkan et.al., 1972, Hanna and Leskowitz, 1973). However, such changes may well affect the potential for macrophage presentation without drastically affecting the overall steric structure of the amino acid, thereby providing at best, a weak argument that the amino acid is not part of the recognized determinant. As indicated in the results section, the amino acid component of either ABA-T or ABA-try is essential to immune recognition leading to positive DTH skin tests or blastogenesis in vitro. A brief, early report showing cross-reactivity between ABA-T primed and ABA-try primed guinea pigs is likely due to the use of another animal species, i.e. a different antigen recognition library, or the use of large ABA-polymer conjugates for challenge

with the possible presence of cross-reactive tertiary structures (Leskowitz, Richerson, and Schwartz, 1970).

The finding that ABA-T and ABA-try caused, in contrast, cross tolerance has interesting implications for possible mechanisms involved in the breaking of ABA-T tolerance with ABA-proteins such as ABA-HGG (Bullock, 1979). Initially, two interpretations of this phenomenon were considered. 1. The new protein carrier recruited new helper cells specific to neoantigens on the ABA-HGG molecule to replace ABA-T specific helper cells which were clonally deleted during the induction of ABA-T tolrance in immature animals. 2. Catabolism of ABA-HGG released ABA- coupled to a variety of amino acids and thus presented new antigens to which the animal was not tolerant. The findings reported here that ABA tolerance was not restricted to the amino acid conjugate used to induce tolerance, argues strongly against the latter possibility. However, earlier reports (Bullock, 1978, Lawn and Leskowitz, 1980) and the <u>in vitro</u> experiments reported here, support the conclusion that at least part of the unresponsiveness of ABA-T tolerized juveniles is the result of active supression which is not amino acid specific. Such cells might be expected to inhibit breaking of tolerance by ABA-HGG, suggesting that suppressor cells, if present, are less effective than HGG specific helper cells. Another possibility is that injecting ABA-HGG into ABA-T tolerant rats does not induce anti-ABA clones at all. Rather, ABA-HGG may induce clones specific to neo-protein determinants caused by unique structural changes in the

protein due to the conjugation of ABA. Similar neo-determinants in the ABA-protein conjugate used to skin challenge, would then induce a skin reaction. Guinea pigs have traditionally displayed such protein determinant immunodominance following injection of hapten conjugated proteins since the classic studies of Benaceraff and Gell (1961). Ray and Ben-Sasson (1979) elegantly analyzed such reactions in guinea pigs and concluded that such neo-antigens were immunodominant when compared to the ABA- structural determinant. Rats however, appear to respond equally well to both ABA and protein determinants. Indeed, the Wistar Furth strain responds almost exclusively to the ABA determinant following priming with ABA-HGG and only poorly to determinants expressed on the HGG molecule (Bullock, 1978).

In conclusion, we have presented evidence that positive immune activity, both DTH skin reactions and in vitro blastogenesis, are both ABA and amino acid specific, i.e. little cross-reactivity was observed between ABA-T and ABA-try. In contrast, both the induction of in vivo tolerance and the suppression of in vitro lymphocyte blastogenesis were completely cross-reactive regardless of the amino acid component used. This suggests that ABA-HGG or catabolic products do not break tolerance by providing new ABA- amino acid antigens.

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FIGURE 1. DTH SKIN REACTIONS TO ABA-AMINO ACID CONJUGATES ARE SPECIFIC FOR BOTH THE ABA AND THE AMINO ACID COMPONENT.

Six Adult Lewis female rats per group were immunized with either 100mcg/animal ABA-T/CFA, 100mcg/animal ABA-try/CFA, or CFA only in the 4 footpads as described in the methods. Twelve days later, animals were skin tested with 100mcg/site PPD, 500mcg/site ABA-T, 500mcg/site ABA-try, and 500mcg/site ABA-39-HGG in 0.1ml of PBS. Twenty-four hours later, skin tests were measured. Results displayed are the average skin reactions (mm diameter), +/- the SEM.

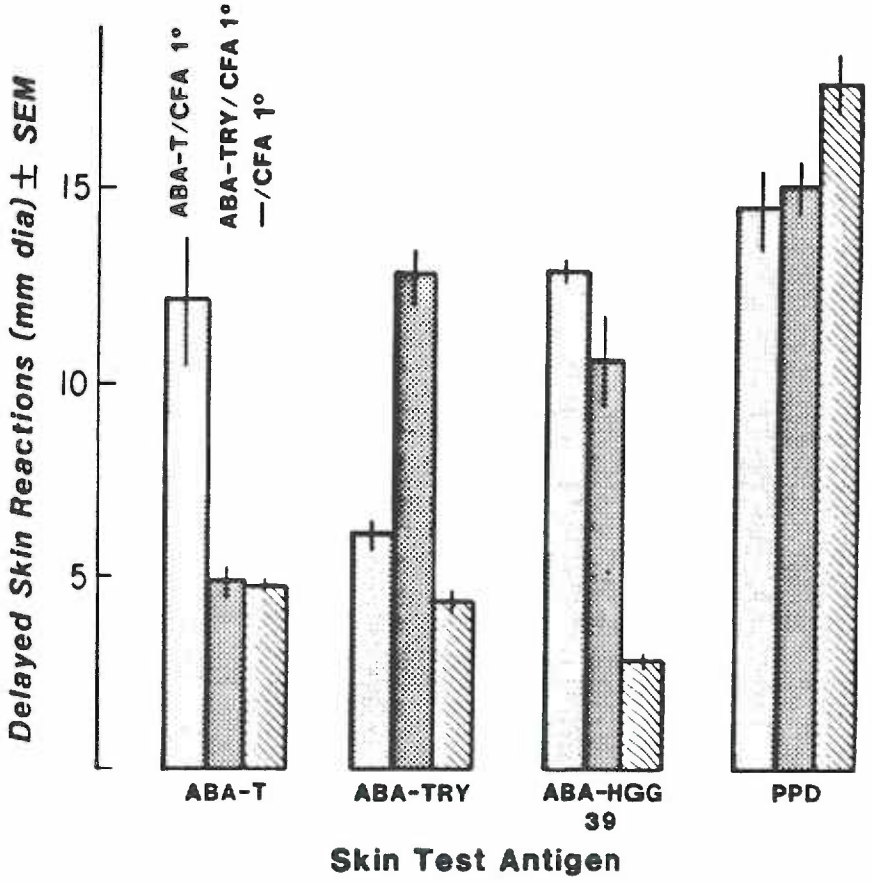

Delayed Skin Reactions (mm dia) ± SEM
15
10
5
ABA-T/CFA 1°
ABA-TRY/CFA 1°
—/CFA 1°
ABA-T
ABA-TRY
ABA-HGG 39
PPD
Skin Test Antigen

FIGURE 2. IN VITRO BLASTOGENESIS REACTIONS TO ABA-TRY IMMUNE CELLS RESPOND MAXIMALLY TO CHALLENGE WITH THE IMMUNIZING ANTIGEN.

Adult Lewis female rats were immunized with 100mcg/animal ABA-try/IFA intraperitoneally as described in the methods. Pooled spleen cells were cultured 20 days after sensitization. Cultures were unstimulated or received 1-1250 mcg/ml of either ABA-try (●——●), ABA-T (●...●), or ABA-39-HGG (O - - O). Cells were cultured for 5 days at which time 0.5mcCi ^{3}H-Thymidine was added. Cells were harvested and counted 24 hours later. Each point is the mean of 6 replicate cultures, +/- the SEM except for the unstimulated culture which is the mean of 12 replicate cultures.

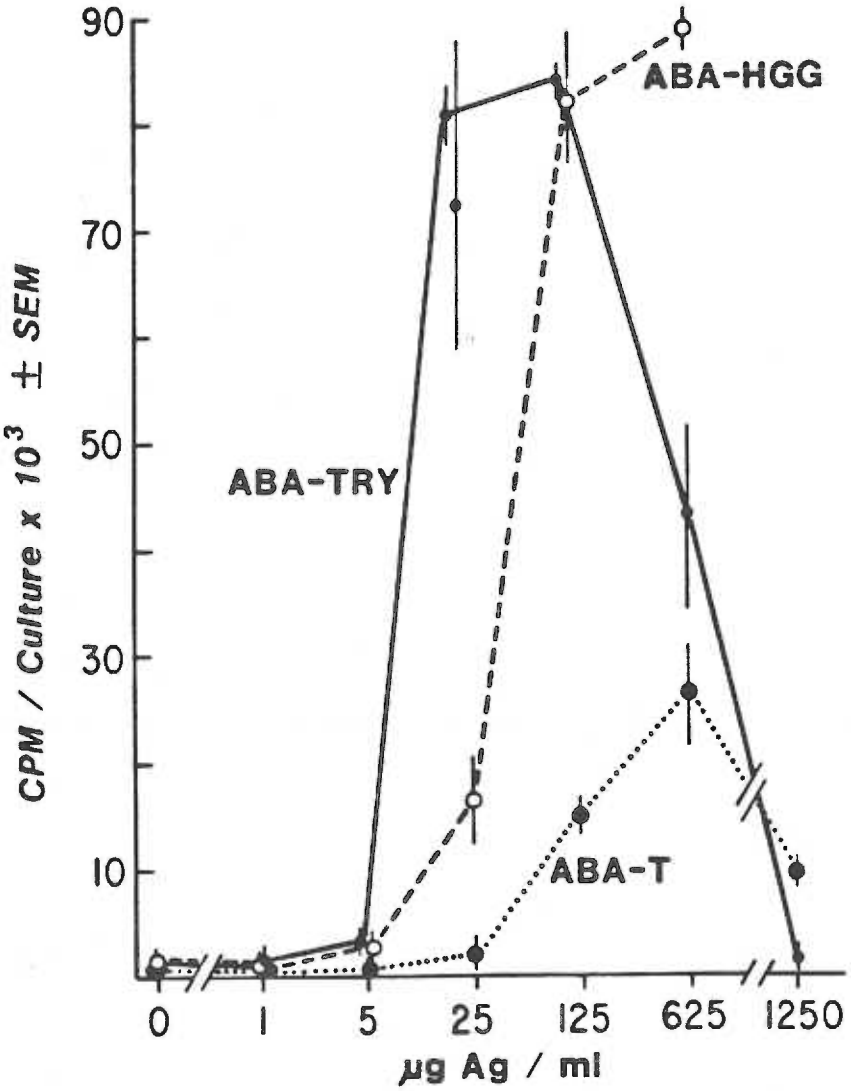

90
70
50
30
10
CPM / Culture x 10^3 ± SEM
ABA-HGG
ABA-TRY
ABA-T
0
1
5
25
125
625
1250
µg Ag / ml

FIGURE 3. IN VITRO BLASTOGENESIS REACTIONS TO ABA-T IMMUNE CELLS RESPOND MAXIMALLY TO CHALLENGE WITH THE IMMUNIZING ANTIGEN.

Adult Lewis female rats were immunized with 100mcg /animal ABA-T/IFA intraperitoneally as described in the methods. Pooled spleen cells were cultured 20 days later. Cultures were unstimulated or received 1-1250 mcg/ml of either ABA-try (●——●), ABA-T (●...●), or ABA-39-HGG (o - - o). Cells were cultured for 5 days when ^{3}H-Thymidine was added. Cells were harvested and counted 24 hours later. Each point is the mean of 6 replicate cultures, +/- the SEM, except for the unstimulated culture which is the mean of 12 replicate cultures.

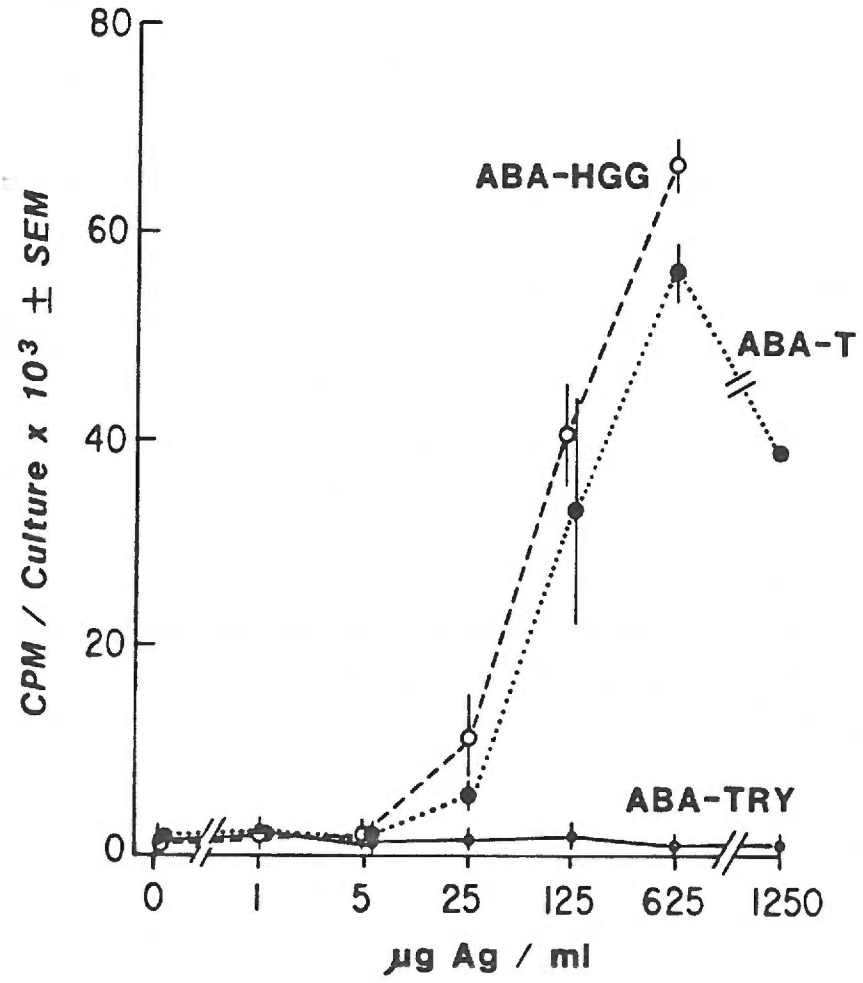

80
60
40
20
0
CPM / Culture x 10³ ± SEM
ABA-HGG
ABA-T
ABA-TRY
0
1
5
25
125
625
1250
µg Ag / ml

FIGURE 4. ABA-T GIVEN TO IMMATURE LEWIS RATS CAUSES SPECIFIC TOLERANCE.

Three week old female Lewis rats were injected in all 4 footpads with IFA only or various doses of ABA-T/IFA. Three weeks after the 1^{o} treatment, all animals received 2^{o} injections of 50mcg ABA-T/IFA in the right footpads and 50mcg HGG/IFA in the left footpads. All animals were skin tested 12 days later with ABA-6-Ovalbumin and HGG, 50mcg each. Reactions were measured 24 hours later. Each point is the mean of 6 animals, +/- the SEM.

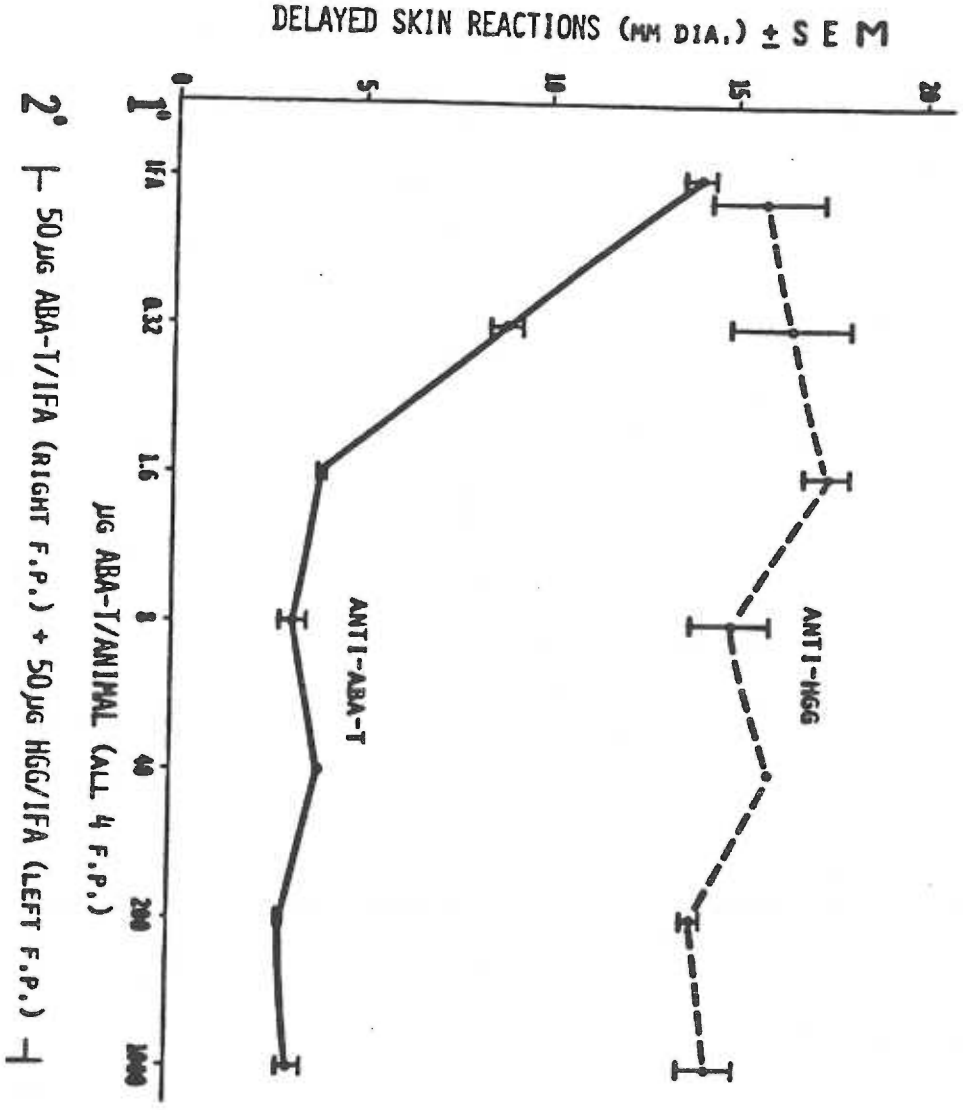

DELAYED SKIN REACTIONS (MM DIA.) ± S E M
0
5
10
15
20
1° IFA
0.32
1.6
8
40
200
1000
μG ABA-T/ANIMAL (ALL 4 F.P.)
ANTI-ABA-T
ANTI-HGG
2° ⊢ 50μG ABA-T/IFA (RIGHT F.P.) + 50μG HGG/IFA (LEFT F.P.) ⊣

FIGURE 5. "JUVENILE" TOLERANCE TO ABA-AMINO ACIDS ARE CROSS-REACTIVE, BLOCKING SENSITIZATION WITH OTHER ABA-CONJUGATES.

Juvenile Lewis female rats, 4 weeks old and 6 animals/group, were given IFA only or 50mcg/animal of either ABA-T/IFA or ABA-try/IFA in the 4 footpads as described in the method. Animals were immunized 4 weeks later with IFA only or 50mcg/animal of either ABA-T/IFA or ABA-try/IFA. Twelve days later, animals were skin tested with 50mcg/site ABA-6-Ovalbumin (shown above) or ABA-28-HGG (not shown) or HGG alone (not shown). Each column is the mean of 6 animals, +/- the SEM. Maximum HGG reaction was < 4mm. ABA-28-HGG produced results comparable to those of ABA-6-Ovalbumin.

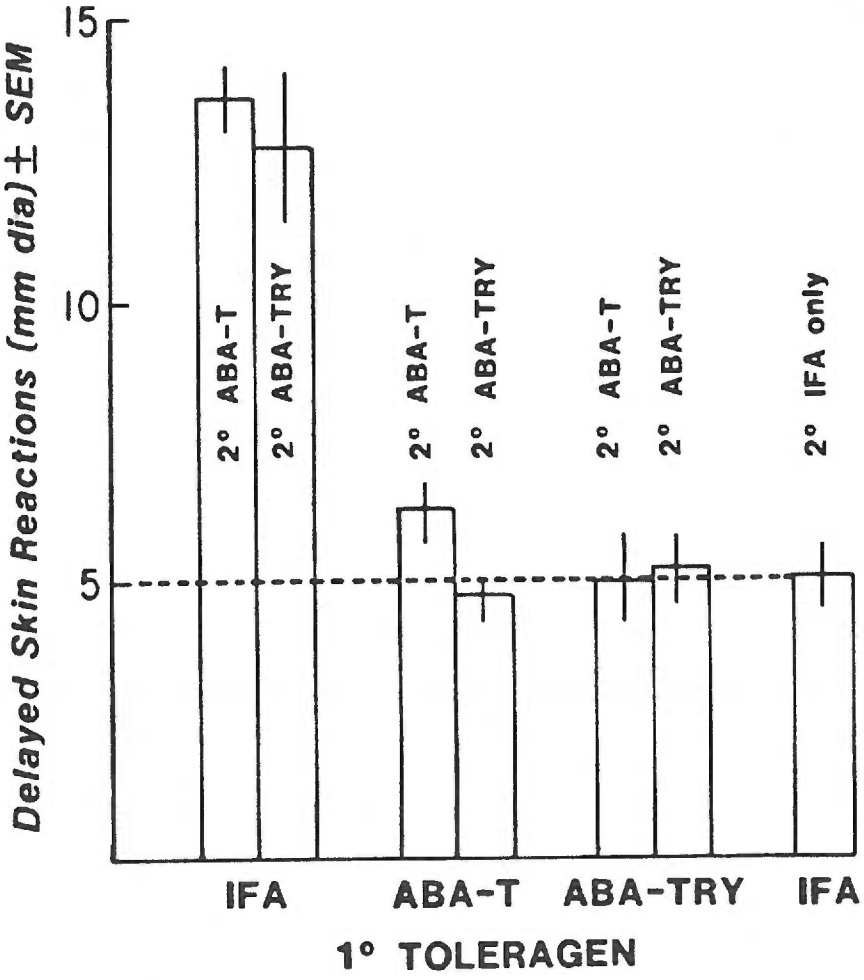

Delayed Skin Reactions (mm dia) ± SEM
15
10
5
2° ABA-T
2° ABA-TRY
2° ABA-T
2° ABA-TRY
2° ABA-T
2° ABA-TRY
2° IFA only
IFA
ABA-T
ABA-TRY
IFA
1° TOLERAGEN

FIGURE 6. ANTIGEN-ACTIVATED SUPPRESSION BLOCKS BACKGROUND TRITIATED THYMIDINE UPTAKE FOLLOWING EARLY SPLEEN CELL CULTURE OF CFA PRIMED LEWIS RATS.

Adult Lewis female rats were immunized with 100mcg ABA-T/CFA in the 4 footpads as described in the methods. Spleen cells were removed and cultured 10 days later. Cultures were unstimulated or received 0.2-125 mcg/ml of either ABA-T, ABA-39-HGG, PPD, or 5 mcg/ml Con A (data not shown). Each point is the mean of 6 replicate cultures, +/- the SEM, except for the unstimulated point which is the mean of 12 replicate cultures.

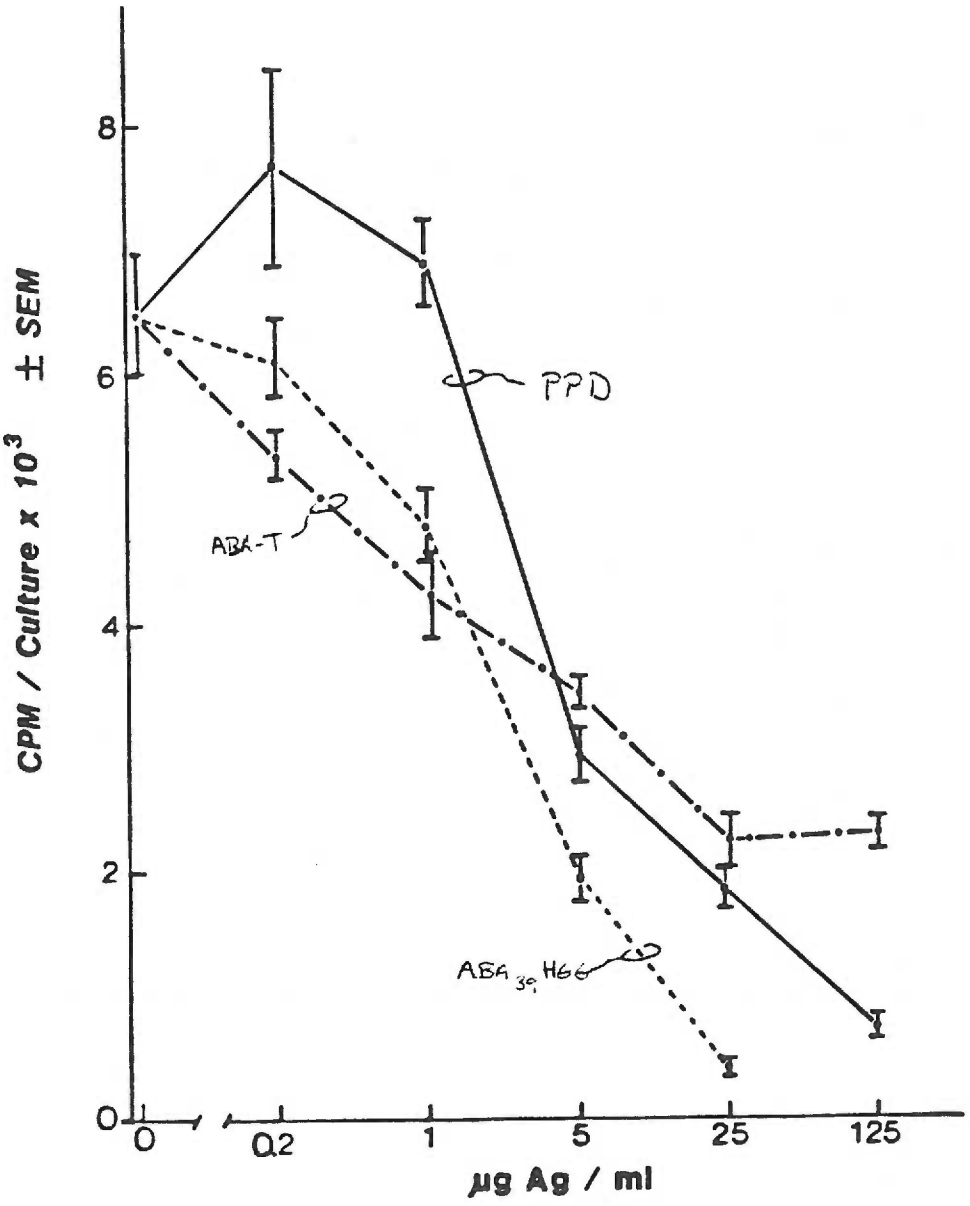
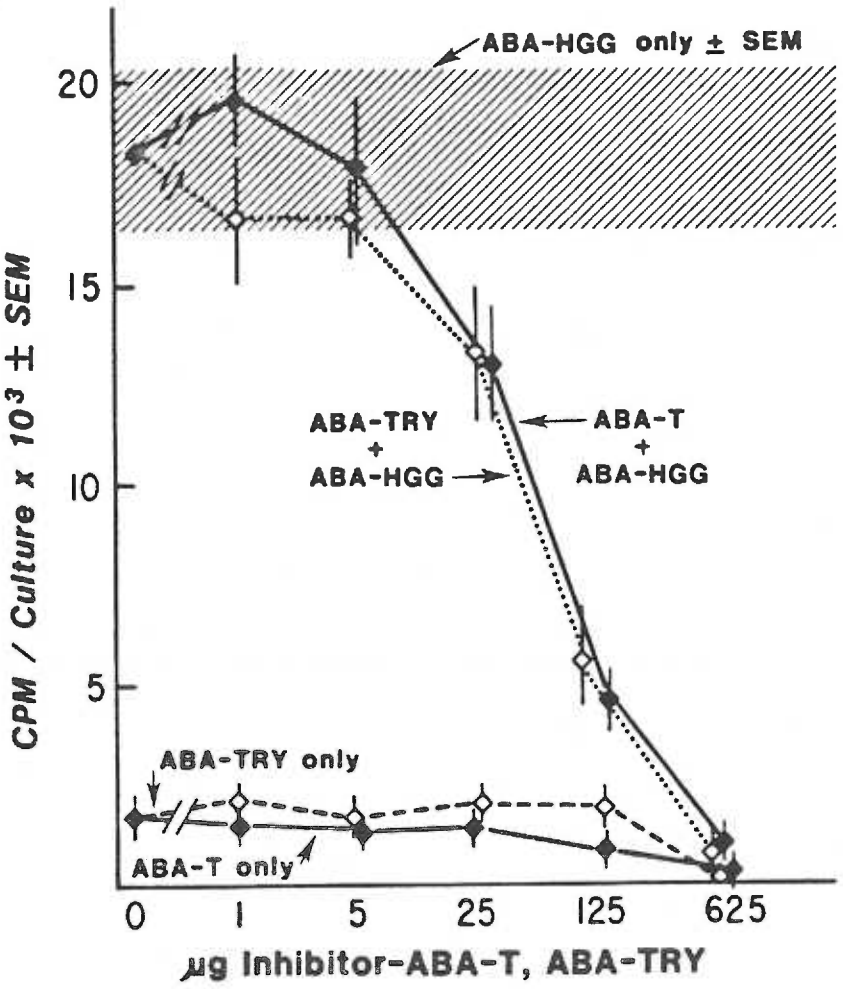

CPM / Culture x 10³ ± SEM
8
6
4
2
0
PPD
ABA-T
ABA 39 HGG
0
0.2
1
5
25
125
µg Ag / ml

FIGURE 7. ABA-T PRIMED SPLEEN RESPONSE TO ABA-39-HGG IS BLOCKED BY EITHER ABA-T OR ABA-TRY IN THE ABSENCE OF CELLS RESPONSIVE TO EITHER ABA-T OR ABA-TRY.

Adult female Lewis rats were sensitized with 100mcg/animal ABA-T/CFA in the 4 footpads as described in the methods. Twelve days later, all animals were skin tested to assure that immunization had occurred (the appropriate positive responses were obtained). Fifteen days later spleen cells fron these animals were cultured without stimulant or with 625 mcg/ml ABA-39-HGG, +/- either ABA-T 1-625 mcg/ml (◆) or 1-625 mcg/ml ABA-try (◇) as shown above. Separate cultures were stimulated with ABA-try 1-625 mcg/ml or ABA-T 1-625 mcg/ml also shown above. The hatched area at the top indicates the response to 625 mcg/ml ABA-39-HGG alone, +/- the SEM.


ABA-HGG only ± SEM
20
15
10
5
CPM / Culture x 10³ ± SEM
ABA-TRY
+
ABA-HGG
ABA-T
+
ABA-HGG
ABA-TRY only
ABA-T only
0
1
5
25
125
625
µg Inhibitor-ABA-T, ABA-TRY

RAPID QUANTITATIVE ANALYSIS OF ACTIVATED INDUCED-PERITONEAL RAT NEUTROPHILS

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ABSTRACT

We present here a rapid, sensitive, and convenient assay for activated Lewis rat PMNs based on detecting separately or in tandom, aggregaton of PMNS and the reduction of nitroblue tetrazolium (NBT). These quantitative assays use an ELISA machine to measure O.D. changes of activated cells (5×10^5 cells/well) in microtiter plates with time. The cellular aggregation and NBT reduction responses can be blocked with either 2-deoxyglucose (0.1 mcg/ml) or iodoacetamide (10mM). The assays are sensitive, detecting aggregation with as little as 0.005 mcg/ml of phorbol myristate acetate (PMA) or 0.01 mcg/ml lipopolysaccharide (LPS) and NBT reduction with as litTle as 0.01 mcg/ml PMA. The LPS-induced aggregation response was inhibited with 10 mcg/ml polymyxin B. In addition, activation of neutrophils was detected following stimulation with lymphokine- containing rat serum diluted up to 1:160 in the aggregaton assay. The convenience of this method for study of rat PMNs is furthered by both the speed with cultures can be studied and the low number of cells required per culture.

INTRODUCTION

Neutrophils require several "skills" to carry out their essential role in protecting the host from the continual threat of bacterial disease. Upon "triggering", PMNs must aggregate to vascular walls and tissue, migrate along a chemotactic gradient, and accelerate oxygen-dependent bactericidal activity. Several disease conditions exist where PMNs lack only some functions while retaining other capabilities. It is not clear if these differences reflect loss of some trigger mechanism or loss of a PMN subpopulation. Unfortunately, the term triggering has frequently been used to imply that PMNs have a single "off/on" switch which activates all of the above PMN activites. Some recent studies however suggest that this may not be true (1-3). If methods can be developed which can conveniently test multiple stimulants over broad dose ranges with reasonable numbers of cells, then we can more fruitfully attack the questions of PMN subpopulations and PMN triggering while gaining insight into diseases associated with PMN defects. However, assays of PMN activity are characteristically complex procedures that require a significant investment in both time and cell numbers. These realities tend to impose rather severe limitations on the number of variables that can be tested at one time employing cells from modest size blood samples.

We recently reported an assay for activation of human PMNs separated from blood, employing an ELISA apparatus to measure O.D. loss which occurs due to cell aggregation. Only small amounts of blood are needed to analyze several hundred separate PMN suspensions (5). However, use of human cells limits study of many variables possible with the use of animal models. We report here that peritoneally-induced Lewis rat neutrophils undergo a similar stimulant-dependent loss in O.D. This activity is blocked by inhibitors of glycolysis, 2-deoxyglucose and iodoacetamide. Although the overall response of rat cells in this assay is similar to that of human cells, the relative activity of various stimulants differs. In addition the variability between experiments is less likely, due to strain homogeneity and a more controlled environment. We have in addition adopted a parallel assay which measures the reduction of nitroblue tetrazolium (NBT) reflecting the "oxidative burst" which follows the activation of neutrophils. This activity is readily detected separately or, for convenience, with the same cell population used to study aggregation.

MATERIALS AND METHODS:

REAGENTS

Lipopolysaccharide B (LPS) Salmonella typhosa 0901 was obtained from Difco Laboratories, Detroit, Michigan. N-formyl-Methionyl-Leucyl- Phenylalanine (F-MET) was obtained from Cal Biochem Behring Corp., San Diego, California. Phorbol myristic acetate (PMA), (Lot 71 FE16104), 2-Deoxyglucose (2-DOG), (Lot 12C-5200), and nitroblue tetrazolium (NBT), (Lot 36C-5034) were obtained from Sigma, St. Louis, MO. Iodoacetamide (Lot 94710) was obtained from Pierce Chemical Co., Rockford, IL.

Preparation of mono p-azobenzenearsonic acid- N- chloroacetyl- L-tyrosine (ABA-T).

500mg p-aminobenzenearsonic acid in 20ml distilled water was dissolved by addition of 3ml of 1N HCL, placed on ice and then 3ml of 5% $NaNO_2$ was added to form the diazonium salt, 1 g of chloroacetyl-L-tyrosine was dissolved in 20ml of 5% Na_2CO_3 and the pH adjusted to 9 with 1N NaOH. The cold diazonium salt of arsanilate was slowly added to the chloroacetyl-L-tyrosine solution and the mixture stirred at room temperature for 2 h maintaining the pH at 9 with 1N HCL and washed. The compound was redissolved and reprecipitated twice from water by bringing the pH to 9 and then

acidifying slowly. The concentration of ABA-T was determined spectrophotometrically using E_{1cm} 1M = 10,500 at 490 nm in 0.1N NaOH (7,8).

Preparation of ABA-protein conjugates.

Conjugates were dialyzed extensively against phosphate-buffered saline (PBS) at 4°C. Final protein concentrations were determined by Lowry protein determination analysis (6) and the degree of ABA substitution on the susceptible amino acid residues was determined spectrophotometrically. Since ABA-lysine does not absorb at 490 nm, it was assumed that the degree of lysine substitution did not significantly affect the calculation of ABA-T/protein (7,8).

Animals and immunization.

Lewis (LEW/f Mai) rats were obtained from Microbiological Assoc., Walkersville, Md. or Charles Rivers Breeding Labs., Wilmington, Mass. and maintained in our breeding colony at the School of Dentistry.

All rats received intraperitoneal injections (IP) of 0.1-0.5ml Equithesin (prepared in our laboratory) as anesthetic prior to sensitization.

Antigens were dissolved in PBS and emulsified with an equal volume of IFA (Difco Labs., Inc.). Antigen emulsions (0.2ml) in IFA (Ag/IFA) were distributed either equally in the four foot pads or in one site IP.

Antigen stimulated serum.

Twenty days after animals were immunized with 100 mcg ABA-T/IFA

given IP, these animals and non-immunized animals, received a systemic challenge via intracardiac injection of 2.0ml (17.6 mg/ml ABA_{59}HGG). Twenty days post-priming was found to be optimal for antigen induced blastogenesis. Thus, this day was chosen for systemic challenge (data not shown). Four hours after receiving the systemic challenge of ABA_{59}HGG, all animals were exsanguinated and the blood was pooled and the serum recovered. The two groups of pooled serum (5-6 animals per group) were then aliquanted and frozen at -70°C until used.

CELL PREPARATION:

Preparation of Human Polymorphonuclear Neutrophils (PMNs) and Overnight Storage.

Human blood was obtained by venipuncture from volunteers and defibrinated by gentle swirling in a flask containing a siliconized steel wool pad. All glassware was siliconized. PMNs were separated using Ficoll-Hypaque discontinuous gradients (9). The isolated fraction typically contained >95% PMNs. The cells in this fraction were washed three times in phosphate buffered saline (PBS) with 10% autologous serum and resuspended in the same medium. Trypan blue exclusion testing usually revealed cell viability to be >99%. PMNs were stored overnight at 1×10^7 cells/ml in a total volume of 8-12ml in (Falcon #2025) 16 x 25-mm plastic tubes in PBS supplemented with 10% autologous serum and a 1% solution of penicillin/streptomycin (50 units penicillin + 50 mcg streptomycin

per 100ml). Tubes were stored at room temperature (between 22-27° Celsius) until used the next day. PMNs were freshly washed, counted, and resuspended in serum, pen/strep, and PBS just prior to use. Although the total number of cells decreased with time, storage did not decrease the percent viability probably due to rapid autolysis of dead cells (data not shown).

Preparation of Rat PMNs and Overnight Storage.

Rat PMNs were obtained by injecting 4-5 female Lewis rats with 20ml of a warm 0.1% oyster glycogen/PBS solution intraperitoneally. Four hours later, the animals were sacrificed, injected IP with 12ml of minimum essential medium (MEM) + 2 units/ml heparin. The peritonel cavity was opened and the peritoneal exudate recovered. Pen/strep was added to the exudate fluid prior to washing the fluid at 200xg for 20 mins at 4 degress C. The supernatant was discarded and the cell pellet resuspended in 10ml of fresh, warm MEM + heparin. Trypan blue exclusion typically revealed >97% viability. Enough autologous serum was added to achieve a 5% solution as well as enough pen/strep to achieve a 1% solution. Cells were stored overnight in 50ml centrifuge tubes at room temperature resting on their sides. The next day, cells were washed, the supernatant was removed and cells cultured +/- stimulant in PBS , 1% pen/strep, and 5% autologous serum. Viability remained better than 97% on the second day. No effort was made to further enrich rat PMNs from the exudate fluid since the population averaged approximately 76% PMNs with a range from 68%-83%.

ASSAY PLATE PREPARATION:

PMNs were assayed in Microtest III plates (Falcon #3072). Routinely, columns 1 and 12 were filled with PBS. The plates were divided into four quadrants and experimental reagents were added to the upper left and lower right quadrants. Cells were not stimulated in the lower left and upper right quadrants. This arrangement minimized experimental artifact due to well location within the plate. Data points were calculated in the following manner. OD values of the upper four wells of column 2 were pooled with the lower four well OD values of column 7 and an average was determined for this particular point in time. The change in OD (delta OD) was calculated by subtracting out the calculated average OD values obtained at "time 0" from values obtained at later time points. "Agent-induced" O.D. is the difference between non-stimulated and stimulated delta OD values. This process was continued throughout the plate with columns 3&8, 4&9, 5&10, and 6&11 representing identical experimental design.

METHODOLOGY OF EXAMINING NBT REDUCTION:

NBT (Sigma Chem. Co.) was disolved in PBS to a final concentration of 2 mg/ml. At the appropriate time, the ELISA filter was adjusted to 630 nm. Each well received 0.025ml of the 2 mg/ml stock solution. Plates were read immediately and various times thereafter.

DATA ANALYSIS AND UTILIZATION:

OD values from the ELISA serial output port were stored in computer disk files on a PDP-11/23 computer. Analysis programs permitted calculation of optical density changes with time, grouping of replicate values, derivation of standared statistics (mean, standard deviation, and standard error), and plotting the results. Responses at individual time points were evaluated with the Mann-Whitney U test; statistical significance of several responses over a period of time was tested with the two-tailed Wilcoxon rank test.

RESULTS

EXPERIMENTAL DESIGN.

The approach used to obtained PMNs from either human peripheral blood or from glycogen-induced peritoneal fluid is graphically shown in Figure 1. Typically, cells from both rats and humans were used the day after the PMNs were collected. Cell cultures are kept at 37° C except when plates were being read on the ELISA machine which required 1-2 minutes.

DECREASED OPTICAL DENSITY OF PMN SUSPENSIONS RESULTING FROM PMA-INDUCED CELL AGGREGATION.

Lewis rat peritoneal PMNs were harvested and stored at room temperature for 24 hours prior to use. Cells were added to microtiter plate wells without stimulant or with 0.005-50.0 mcg/ml PMA. Plates were read with an ELISA machine and then incubated at 37°C between each reading. As shown in Figure 2 the control suspensions underwent modest changes in optical density due to the intial settling out and spreading of cells. It can be seen that a rapid, dose dependent loss in O.D. occurred with PMA treated cells compared to the controls. It is also clear that the high 50 mcg/ml PMA dose produced extensive loss in O.D. likely reflecting actual damage to cells since this is a toxic dose (10).

AGENT-INDUCED LOSS OF O.D. IS A RAPID REPRODUCIBLE METHOD TO DETECT PMN AGGREGATION.

Figure 3 presents the mean of values obtained with four consecutive experiments similar to that described in Figure 2. Results are presented with the O.D. changes of non-stimulated cultures subtracted out to better visualize those changes due to the PMA activity. Again it can be seen that the changes are rapid and dose dependent. The assay is also extremly sensitive, detecting as little as 0.005 mcg/ml PMA. All responses are significant over the time priod tested ($p < 0.001$) as determined with the Wilcoxin-rank test.

EARLY KINETICS OF PMA INDUCED AGGREGATION.

It is known that aggregation is an early event following PMN stimulation. Within the limits of the time required to add cells to the plate we have consistantly observed early changes in O.D. starting within 1-2 minutes and peaking within 10 minutes (Figure 4). Later experiments with cells added first, followed by addition of stimulant indicate that we are detecting the majority of early O.D. loss (not shown). These slower than normal kinetics are likely due to cells settling out on the bottom of the wells.

DEPENDENCY OF PMA-INDUCED AGGREGATION ON GLYCOLYTIC PATHWAY.

We know from earlier work in our laboratory that glutaraldehyde

treated PMNs do not undergo stimulant-induced loss in O.D. However, the possibility remained that this treatment may have modified some surface component essential to some form of "physical aggregation". To further insure that we were measuring biologically relevant activation we determined the need for an energy-dependent metabolic step. Rat PMNs were stimulated with 0.005-5.0 mcg/ml PMA in the presence or absence of 10 mM 2-deoxyglucose, an inhibitor of glycolysis. As shown in Figure 5, glycolysis was essential to PMA-induced loss of O.D. We have repeated this experiment, with similar results using human PMNs (Figure 6). In addition, a second inhibitor, 10mM iodoacetamide, produces a similar suppression of aggregation (Figure 9).

LPS-INDUCED AGGREGATION OF RAT PMNs.

We have previously reported that LPS is a potent initiator of human PMN aggregation in this assay (5). As shown in Figure 7, LPS was less effective than PMA in lowering the O.D. of rat cells. Furthermore, human PMNs responded to as little as 0.0001 mcg/ml of LPS making the rat response less sensitive as well. To insure that this low response in rats was due to LPS activation, an inhibitor of the biologically active lipid A component of LPS, polymyxin-B, was added. As can be seen, polymyxin B abrogated the stimulation due to LPS.

ACTIVATION OF RAT PMNs BY ANTIGEN-INDUCED LYMPHOKINE(S).

As previously reported large levels of lymphokine are obtainable by systemic challenge of immune animals (4). We therefore challenged normal rats and rats immunized with ABA-T, an antigen known to selectively immunize T-cells, 20 days post-priming with ABA_{59} -human gamma globulin-conjugate antigen I.V. Four hours later animals were bled and the serum pooled, and stored at -70°C until used. Sera were assayed at dilutions indicated in Figure 8 for PMN activation. As can be seen, the pooled, putative lymphokine preparation immune serum was a potent activator, giving strong stimulation even when diluted 1:160. The control serum lacked PMN stimulating activity.

REDUCTION OF NITRO-BLUE TETRAZOLIUM (NBT) BY PMA-ACTIVATED RAT PMNS.

Pick et al. (3) recently reported the NBT reduction by monocytes can be measured with the ELISA machine. We therefore stimulated rat PMNs, with or without 10 mM Iodoacetamide, with 1 mcg/ml PMA for one hour at 37°C. NBT (0.025 ml of a 2 mg/ml solution or 0.05 mg/well total) was then added to each well and plates were read at 0, 1, and 2 hours later to detect for the presence of reduced NBT (630 nm). Thus, the ordinant = the O.D. obtained for normal cells minus the O.D. obtained for iodoacetamide treated cells. As shown in Figure 9, stimulated cells rapidly reduced the NBT indicating triggering of the PMN "oxidative burst". The nonstimulated cells were no nore active than cells treated with iodoacetamide which blocks PMN's glycolytic pathway.

SEQUENTIAL MEASUREMENTS OF THE SAME PREPARATION OF PMA-ACTIVATED RAT PMNS FOR AGGREGATION AND NBT REDUCTION.

Rat PMNs were stimulated with three different doses of PMA (0.01, 0.1, and 1.0 mcg/ml; two 1.0 mcg/ml PMA doses were studied at two different locations in the plate to look at positonal vatiation), and aggregation was determined for the next two hours. Figure 10 shows a dose dependent aggregation response to the concentrations of PMA present in the culture. At the conclusion of the aggregation measurements, the filter on the ELISA spectrophotometer was changed from 410nm to 630nm. Then, 0.025 ml of a 2 mg/ml NBT solution was added to each well and the plate read immediately after the addition of the NBT. Except for the time necessary to add the NBT solution and read the plate, the plate was maintain at 37^{o} C for the next two hours and read at the time intervals shown in Figure 11. Strong reduction of NBT was observed with the same cells used originally for the study of aggregation. Thus, the same cell preparation can be assay for two PMN activities. It was found that testing rat PMNs for their ability to reduce NBT without looking at aggregation first, showed essentially the same rate of reduced NBT production (data not shown). This suggested that rat PMNs generate superoxide slowly, taking 2-3 hours to produce enough superoxide to achieve the maximum amount of reduced NBT in an experiment. This contrasts with human PMNs which produces large amounts of superoxide within minutes after stimulation with

PMA (manuscript in preparation).

The aggregation activity produced small O.D changes, and was therefore subject to greater variation induced by subtle differences in well position, temperature, and plate agitation. Thus, the same plate occassionally produced slightly different curves for a given concentration of reagent in the rat system. This is shown in figure 10 where the same dose of PMA (1.0 mcg/ml) is examined in two different locations of the plate. The larger O.D. changes obtained with NBT reduction overcomes this degree of variation (please compare Figure 10 to Figure 11).

DISCUSSION

This report details the isolation and analysis of Lewis rat PMNs, presenting useful, rapid, and sensitive methods to study these cells. With these assays one rat supplies enough cells to analyze 100 separate cell suspensions.

Neutrophils become sticky following activation. It has been proposed that this phenomenon plays a role in adherence of PMNs to the vascular endothelium. This binding activity can be detected *in vitro* either by cell adherance to nylon wool (11), or cell-cell aggregation, by counting aggregate particles (12), or decreased optical density of a PMN suspension (13,14). Although extremely useful, such assays usually require large numbers of cells permitting only a limited number of variables to be tested at any one time. We present here a quantitative assay for rat PMN activation based on the aggregation-induced loss of O.D. due to decreased light scatter. The technique is based on an earlier approach, the use of light transmission to measure aggregation of stimulated platelets (15). Craddock et al., modified this approach to study activation of PMN by C5a, and determined that PMN aggregation can be detected within minutes as measured by decreased O.D. of a PMN suspension in a "modified" cuvette (13,14). We subsequently were able to miniaturize and automate this approach to

study human blood. PMNs (5).

Using this assay we report here that rat PMNs could be activated by as little as 0.005 mg/ml PMA or 0.01 mg/ml LPS. In addition, serum from antigen-activated immune animals was able to stimulate cells after 1:160 dilution. Blockade of the glycolytic pathway with either 2-deoxyglucose or iodoacetamide prevents aggregation indicating a central role for glycolysis. Stimulation with LPS was also blocked by the known LPS inhibitor, polymyxin-B. Although the overall response of rat PMNs was similar to that of human cells, the response to LPS was smaller. This could reflect a difference in cell activity or a difference in the extent to which the respective species are able to provide reactive complement products in the presence of LPS. The longer time required to detect LPS stimulation, compared to the rapid PMA activation, may also reflect the rate of complement product formation or differences in trigger mechanisms.

In summary, we have developed two assays useful in detecting seperate activities of rat neutrophils. The first acitivity is an aggregation event believed to mimic margination of neutrophils to blood vessels prior to their exiting the vasculature. This detected event occurs rapidly and shows dose response to various stimulators. The event is energy dependent since both 2-deoxyglucose and iodoacetamide can block this activity.

The second activity measures the ability of stimulated rat neutrophils to reduce nitroblue tetrazolium, an event believed to

measure the "oxidative burst" of neutrophils. This event is also dose responsive to PMA stimulation and can be inhibited with iodoacetamide. The assay is a rapid, quantitative assay relatively simple to conduct and adaptable for at least two rat neutrophil activities.

Finally, we provide here evidence that a factor(s) is detectable in the sera of rats immunized and challenged systemically with a potent T-lymphocyte antigen. This is preliminary evidence for the existance of T-cell lymphokines that can stimulate at least the aggregation activity of rat neutrophils.

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14. Craddock, P.R., White, J.G., Weisdorf, D.J., Hammerschmidt, D.E., INFLAMMATION: 4: 381-194, (1980).
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16. Klempner, M.S. and Gallin, J., BLOOD: 51: 659-669, (1978).

FIGURE 1. EXPERIMENTAL DESIGN. A graphic description of how PMNs were obtained and assayed. PMNs were typically maintained overnight in medium as described in Materials and Methods. The next day cells were added to Microtest III plates +/- stimulant, kept at 37° C. and intermittantly scanned over a period of hours using an ELISA spectrophotometer at 410 nm or, following addition of NBT, 630 nm. The stored data was analysised on a PDP-11 minicomputer which calculated the stimulatant-induced changes of O.D.

NEUTROPHIL ACTIVATION ASSAY

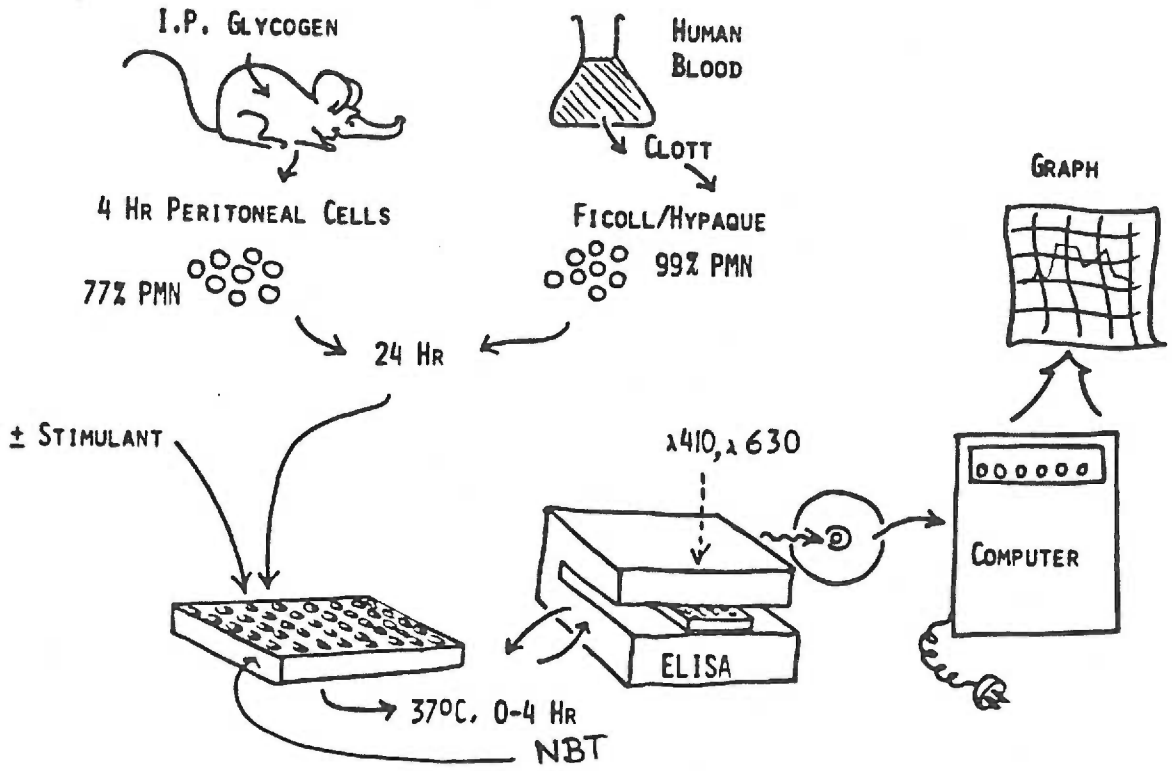


FIGURE 2. DECREASED OPTICAL DENSITY OF PMN SUSPENSIONS RESULTING FROM PMA-INDUCED CELL AGGREGATION. LEW rat peritoneal PMNs were harvested and stored at room temperature for 24 hours prior to use. Cells were added to micrtiter plate wells without stimulant or with 0.005-50.0 mcg/ml PMA. Between readings, plates were kept at 37° C. Each point represents the mean of O.D. differences observed between the start of assay and the time shown for each of eight cultures. Each experimental point is depicted within the figure as the negative $\log_{10}$ of the PMA concentration in mcg/ml of culture; thus 50.0, 5.0, 0.5, 0.05, and 0.005 mcg PMA/ml = ▲, ○, ●, △, ■ respectively, and control (PBS only) = (□).

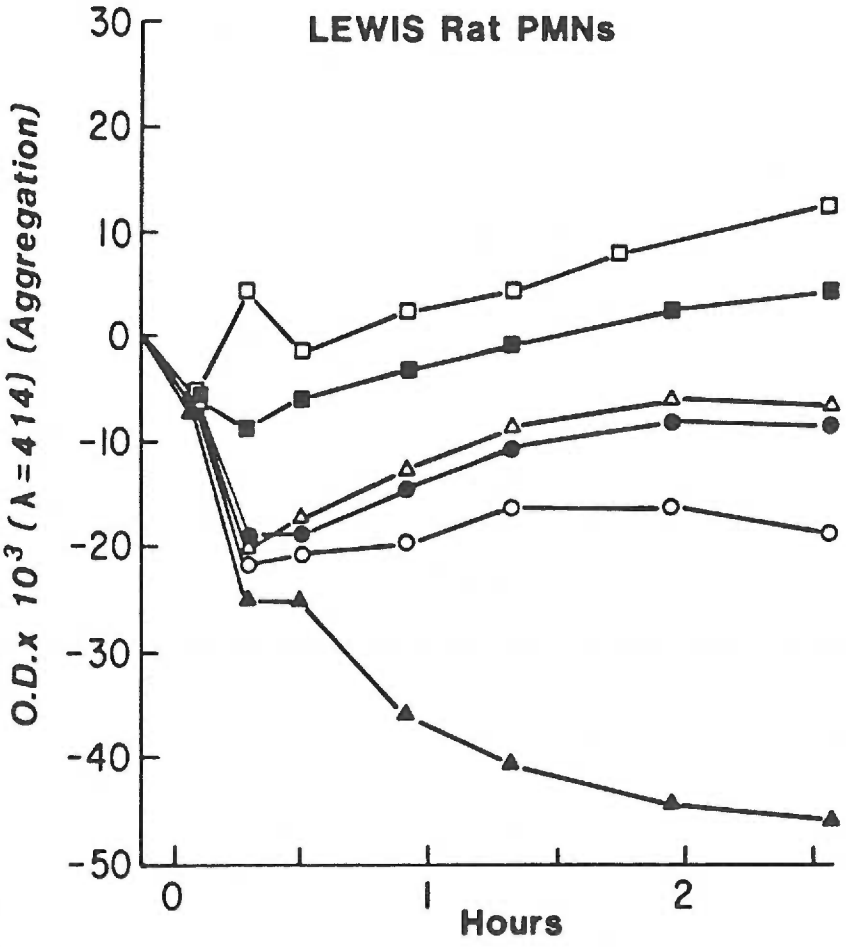

LEWIS Rat PMNs
O.D.x 10³ (λ=414) (Aggregation)
30
20
10
0
-10
-20
-30
-40
-50
0
1
2
Hours

FIGURE 3. AGENT-INDUCED LOSS OF O.D. IS A RAPID REPRODUCIBLE METHOD TO DETECT AGGREGATION OF RAT INDUCED-PERITONEAL PMNS. Pooled data from four separate experiments are depicted. The ordinate represents the O.D. of PMN plates treated with 5.0, 0.5, 0.05, or 0.005 mcg PMA/ml minus the O.D. of controls (PBS only). The abcissa shows the 0 to 3 hour time course studied. Each point is the mean value of the four experiments, +/- SEM.

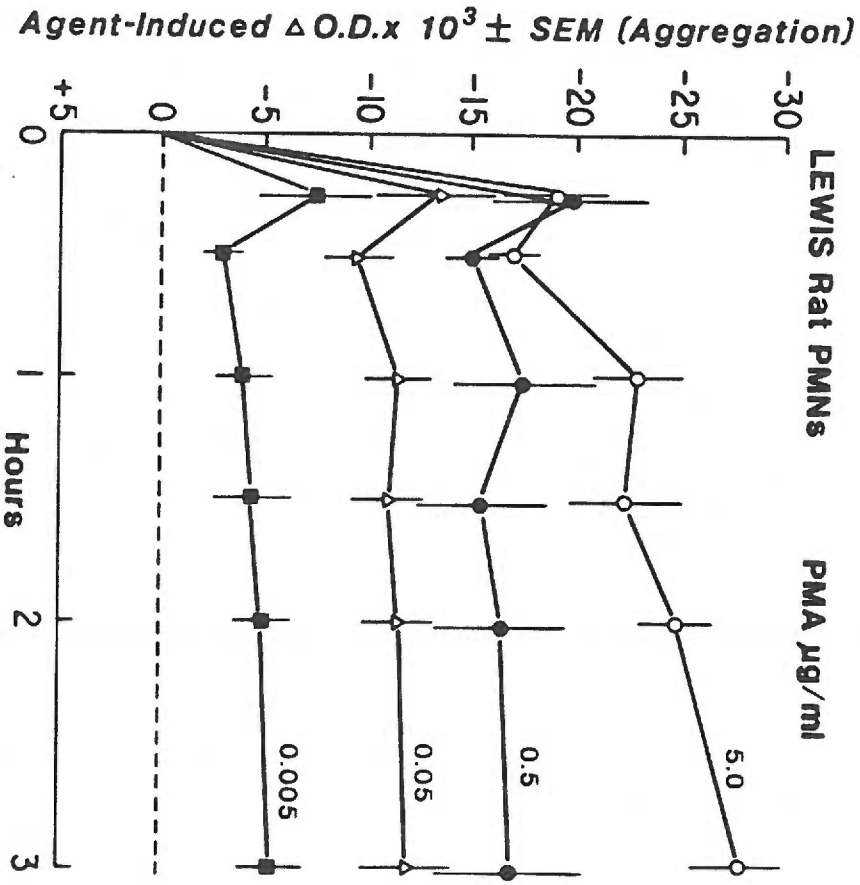

Agent-Induced Δ O.D.x 10³ ± SEM (Aggregation)
+5
0
-5
-10
-15
-20
-25
-30
LEWIS Rat PMNs
PMA µg/ml
5.0
0.5
0.05
0.005
0
1
2
3
Hours

FIGURE 4. EARLY KINETICS OF PMA-INDUCED AGGREGATION. Rat PMNs were treated with 0.5 mcg/ml PMA. The results are plotted as in Figure 3. Thus, the values plotted represents stimulation obtained with 0.5 mcg/ml PMA with the reponse of non-stimulated cells subtracted out.

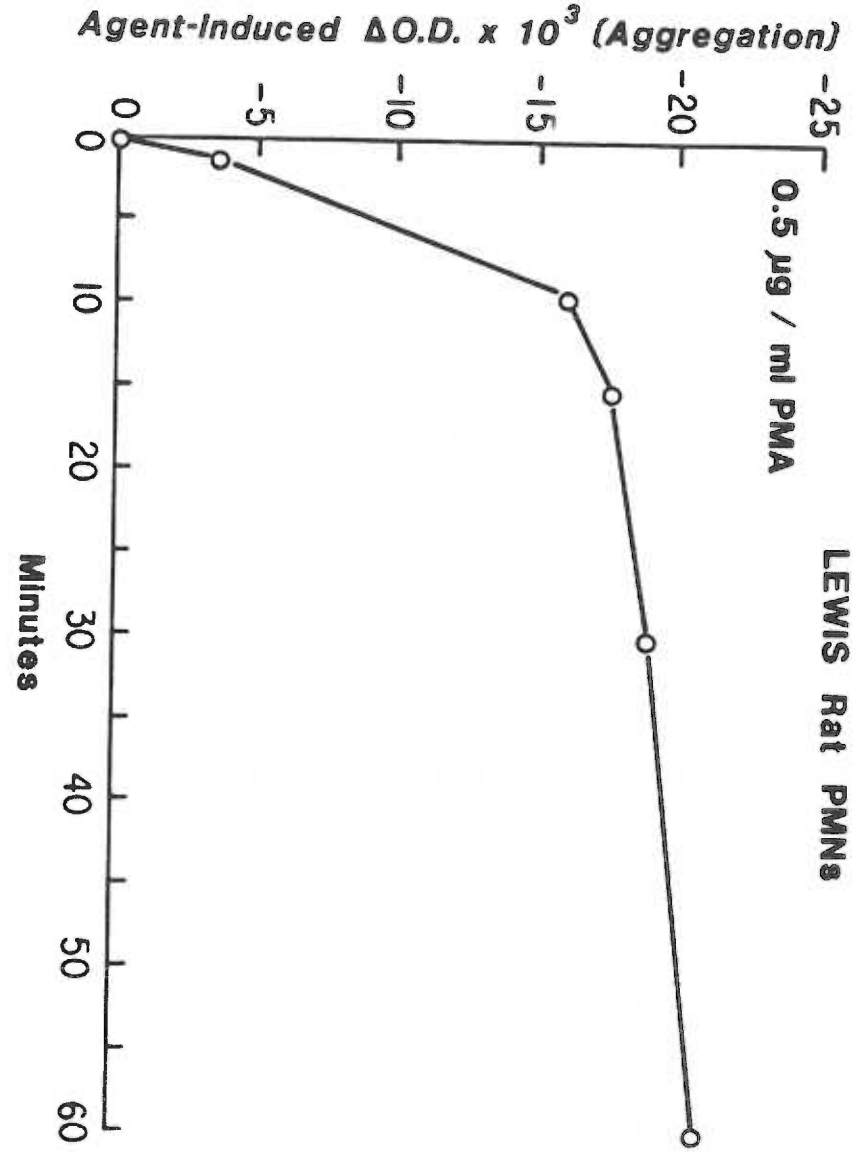

Agent-Induced ΔO.D. x 10³ (Aggregation)
0
-5
-10
-15
-20
-25
0
10
20
30
40
50
60
Minutes
0.5 µg / ml PMA
LEWIS Rat PMNs

FIGURE 5. EFFECT OF 2-DEOXYGLUCOSE (2-DOG) ON PMA-STIMULATED PMNS. PMNs were added to wells without stimulant or with 0.005-5.0 mcg/ml PMA. A) without 2-DOG, B) with 10mM 2-DOG. The results are presented as in Figure 3 and represents the stimulaton obtained with the agent minus the response of non-stimulated cells.

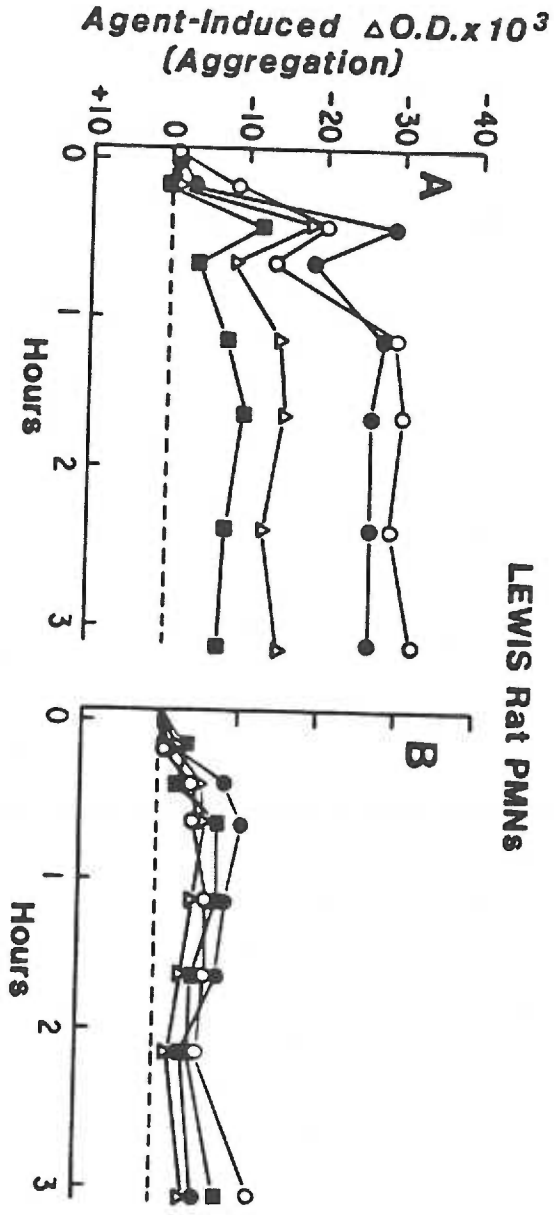

Agent-Induced ΔO.D.x10³
(Aggregation)
+10
0
-10
-20
-30
-40
A
B
Hours
0
1
2
3
LEWIS Rat PMNs

FIGURE 6. EFFECT OF 2-DEOXYGLUCOSE (2-DOG) ON PMA-STIMULATED HUMAN PMNS. A representative experiment similar to Figure 5 but using human PMNs. Human PMNs were stimulated with either 0.1 or 1.0 mcg/ml PMA, with or without 10 mM 2-deoxyglucose present in the culture. Responses shown represent stimulation obtained minus the reponse of non-stimulated cells.

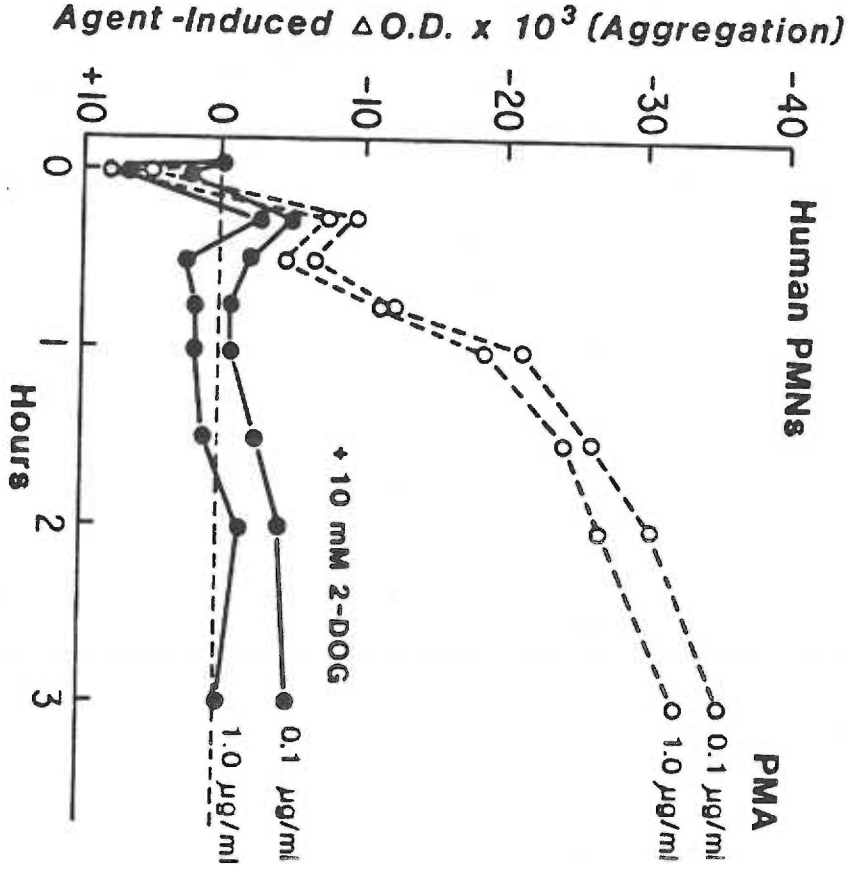

Agent-Induced ΔO.D. x 10³ (Aggregation)
+10
0
-10
-20
-30
-40
Human PMNs
PMA
0.1 µg/ml
1.0 µg/ml
+ 10 mM 2-DOG
0.1 µg/ml
1.0 µg/ml
0
1
2
3
Hours

FIGURE 7. RESPONSE OF RAT PMNS TO LPS , +/- POLYMYXIN-B. Rat PMNs were cultured without stimulation or with 0.1-10.0 mcg/ml LPS present at the initiation of the experiment. The open circles are cultures without polymyxin-B present and the close circles are cultures with polymyxin-B present. The results are presented as in Figure 3.

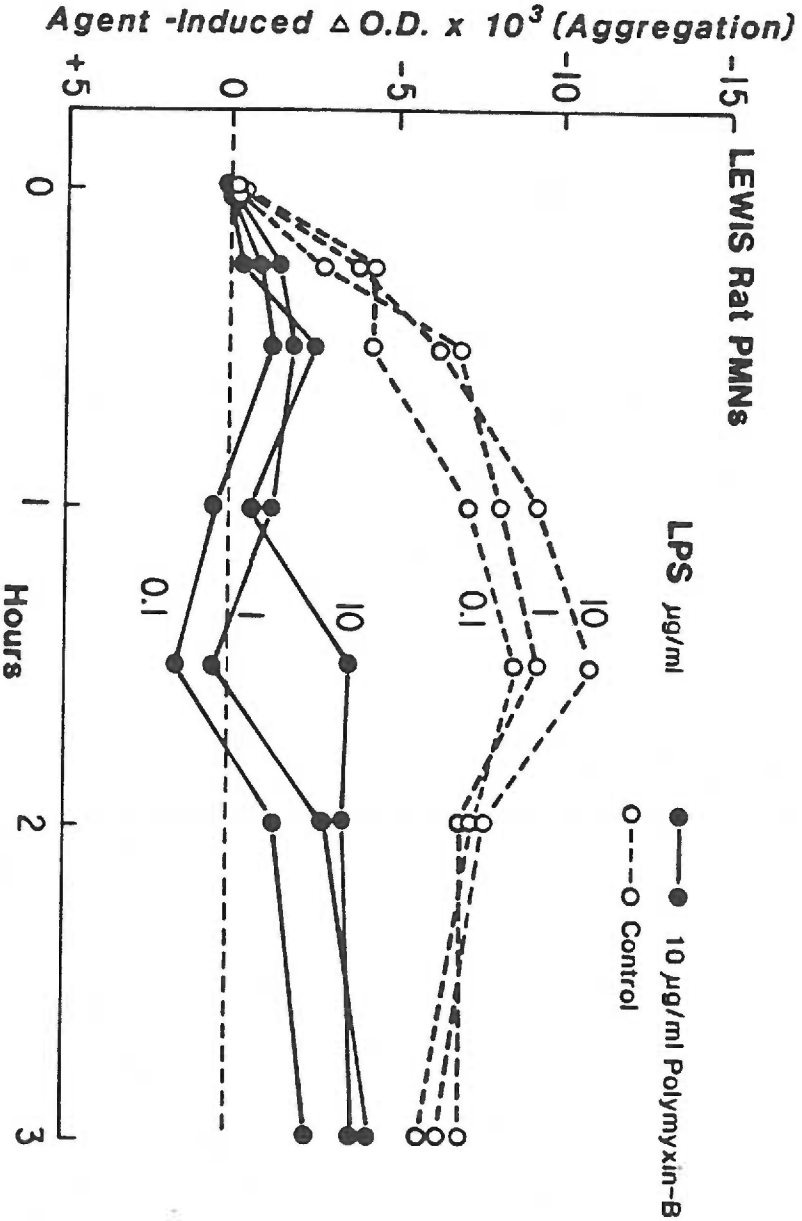

Agent -Induced Δ O.D. x 10³ (Aggregation)
+5
0
-5
-10
-15
LEWIS Rat PMNs
0
1
2
3
Hours
0.1
1
10
LPS µg/ml
10 µg/ml Polymyxin-B
Control

FIGURE 8. Effects of serum obtained from ABA-T immunized animals challenged 20 days post-priming with ABA_{59} -HGG systemically via intracardiac injection. Four hours later, animals were exsanguinated and the serum was collected and pooled. Control serum was obtained identically as Immune serum except the animals were not immunized (see Materials and Methods for further details). The ordinate shows Agent Induced O.D., while the abcissa shows the time course studied. The left panel shows the effects of three dilutions of Immune serum, the right panel shows the effects of three dilutions of Control serum on the ability to cause PMN aggregation. Results are plotted as shown in Figure 3 with serum stimulated responses minus the responses obtained from non-stimulated cells.

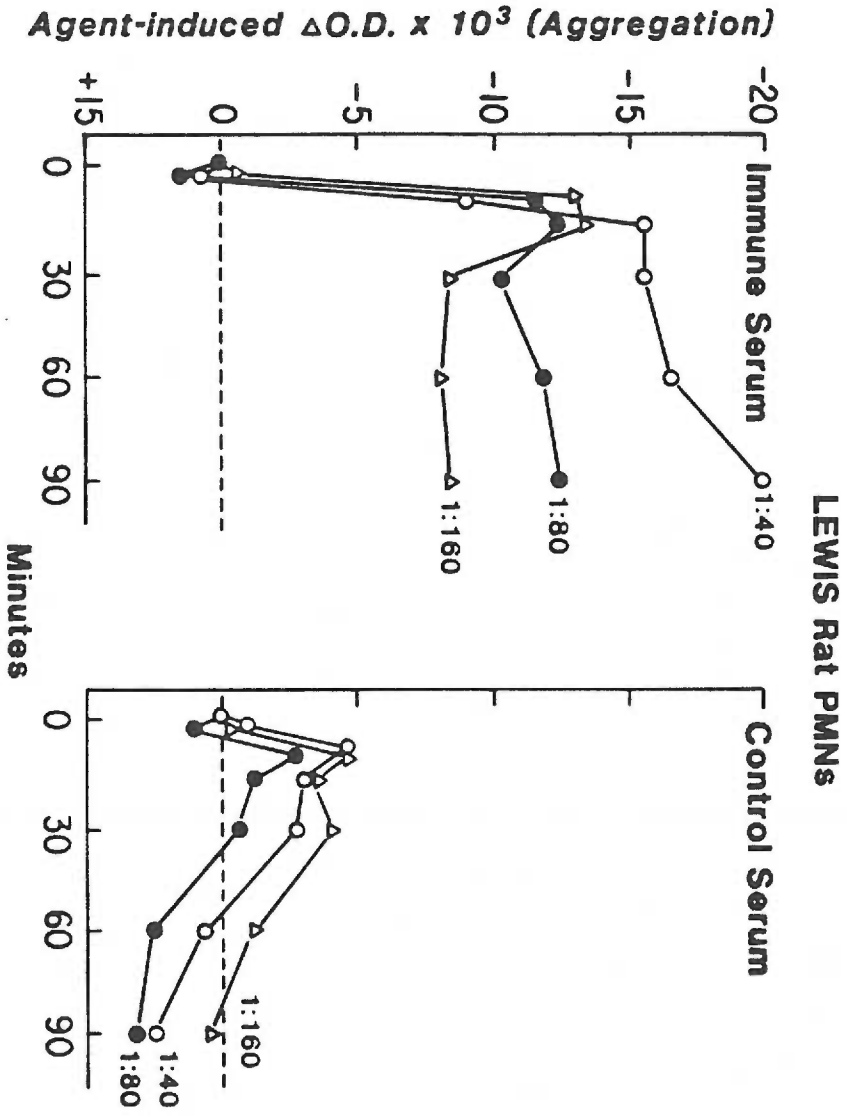

Agent-induced ΔO.D. x 10³ (Aggregation)
LEWIS Rat PMNs
Immune Serum
Control Serum
-20
-15
-10
-5
0
+15
0
30
60
90
Minutes
1:40
1:80
1:160

FIGURE 9. Reduction of Nitro-Blue Tetrazolium (NBT) by PMA activated PMNs. The ordinate shows the O.D. obtained from PMNs minus the O.D. obtained from blocked PMNs (PMNs treated with 10 mM Iodoacetamide 10 mins. before addition of PMA). The abcissa shows the time course studied. At -10 mins., the first reading was taken with cells present with or without iodoacetamide and the readings were used to establish the "zero" Agent-induced line (i.e. the values used as "blanks"). At time 0, PBS or 1 mcg/ml PMA was added to the appropriate cultures. At time 1 hr., NBT was added to all wells. ■ = cells + PBS (negative control); □ = cells + 1 mcg/ml PMA.

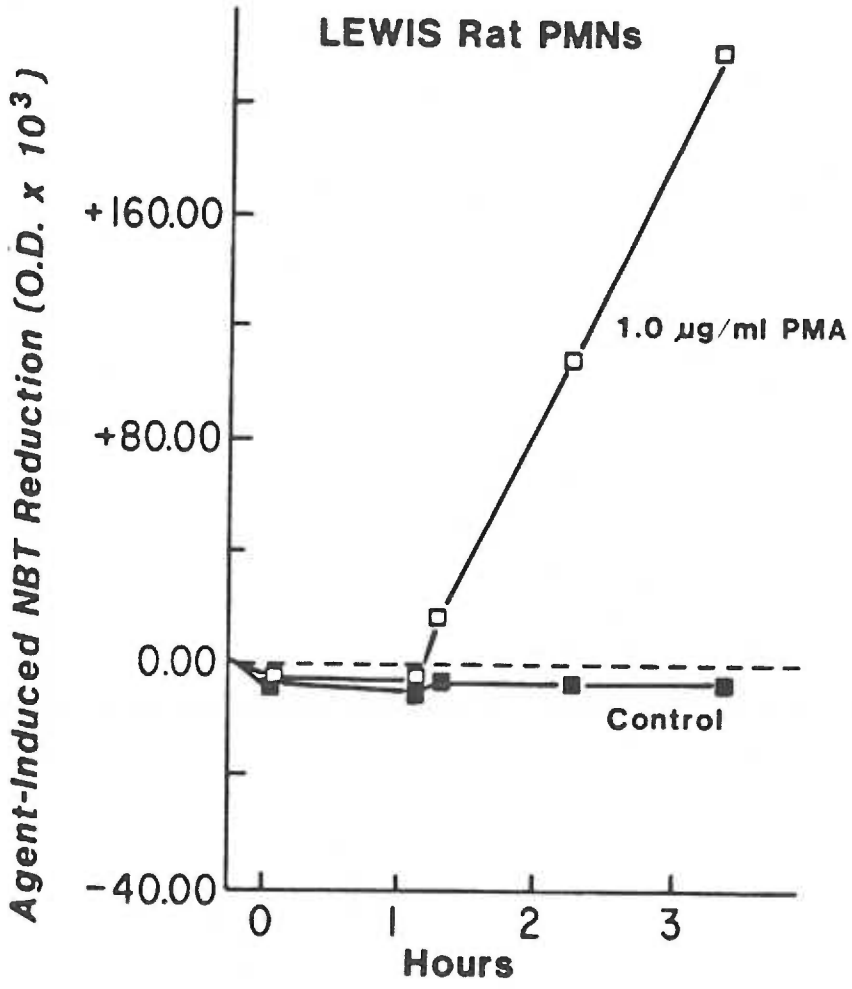

LEWIS Rat PMNs
Agent-Induced NBT Reduction (O.D. x 10³)
+160.00
+80.00
0.00
-40.00
1.0 µg/ml PMA
Control
0
1
2
3
Hours

FIGURE 10. AGGREGATION OF PMA-ACTIVATED RAT PMNS.

As a two part experiment examining if PMA-activated rat PMNs could be first measured for aggregation then tested for their ability to reduce NBT, rats were stimulated with at the initiation of the study with 1.0 - 0.01 mcg/ml PMA. Shown here are rat neutrophils aggregating due to PMA activation. Results are plotted as in Figure 3. Two series of cells were stimulated with the same dose of PMA (1.0 mcg/ml) to look at postional effects in the culture plate.

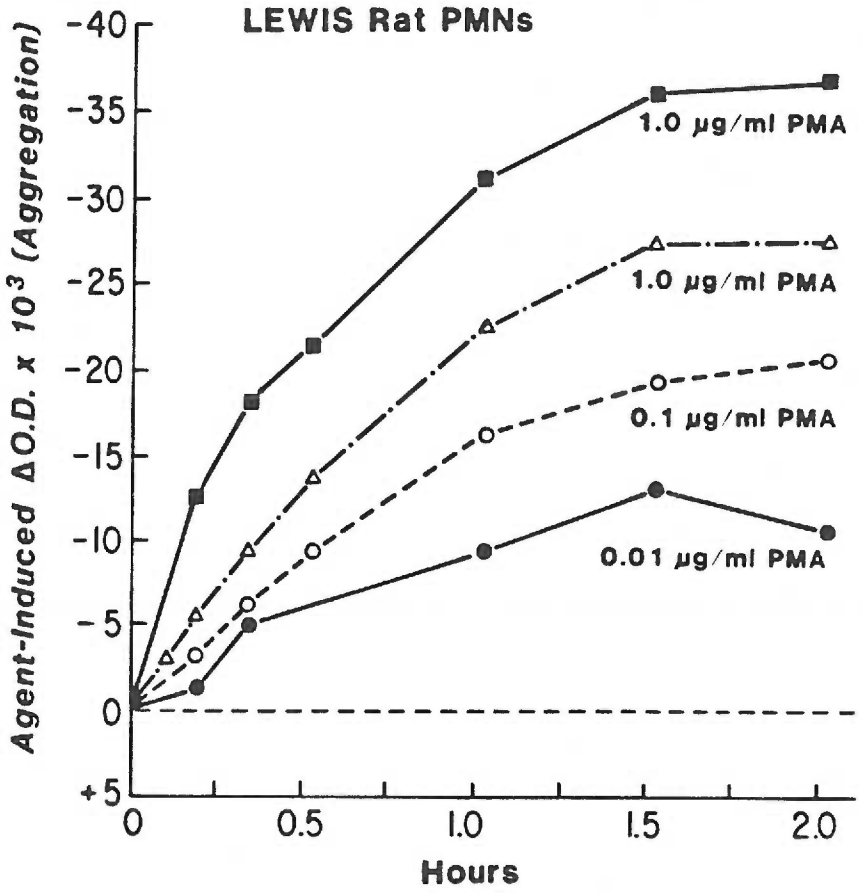
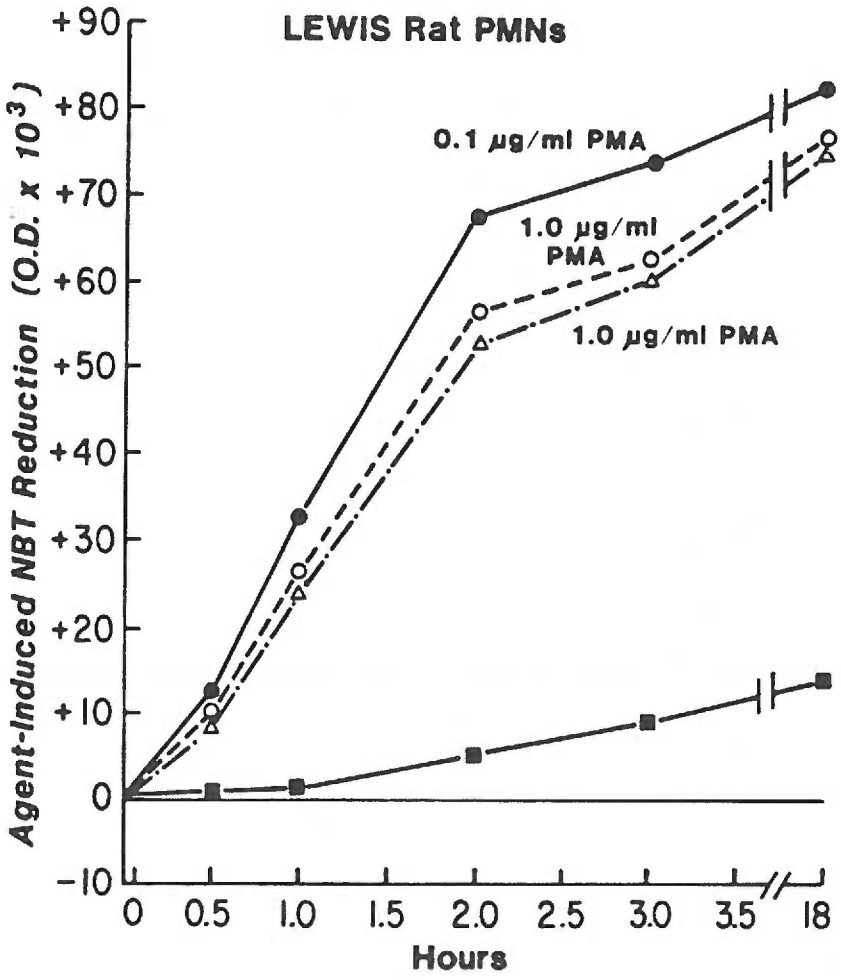

LEWIS Rat PMNs
Agent-Induced ΔO.D. x 10³ (Aggregation)
-40
-35
-30
-25
-20
-15
-10
-5
0
+5
0
0.5
1.0
1.5
2.0
Hours
1.0 µg/ml PMA
1.0 µg/ml PMA
0.1 µg/ml PMA
0.01 µg/ml PMA

FIGURE 11. REDUCTION OF NBT BY PMA-ACTIVATED RAT PMNS AFTER THE CELLS WERE FIRST STUDIED FOR AGGREGATION ACTIVITY.

Rat PMNs were first studied for aggregation due to PMA activation (see Figure 10). Immediately after the termination of the aggregation study, 50 mcg of oxidized NBT was added to all wells. The ELISA spectrophotometer filter was adjusted to 630 nm. Readings began at that point in time, approximately 140 minutes after the initiation of the cultures. Results are plotted as depicted in Figure 9. Thus, the response of PMNs to reduce NBT are shown with the degree of NBT reduction from non-stimulated PMNs subtracted out. As in Figure 10, two series of cells were stimulated with the same dose of PMA (1.0 mcg/ml) to look at positional effects in the culture plate.


LEWIS Rat PMNs
Agent-Induced NBT Reduction (O.D. x 10³)
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
0.1 µg/ml PMA
1.0 µg/ml PMA
1.0 µg/ml PMA
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
18
Hours

THESIS DISCUSSION

An initial goal of this study was to ascertain the minimum size of the antigen complementarity site of the T-cell receptor. To do this, it was decided to examine the role of the amino acid present in the small but complete T-cell antigen ABA-T. We asked if the amino acid contributes to the recognition complementarity of the antigen receptor displayed on ABA-T immune T-cells. To ask this question, another ABA- amino acid, ABA-try, was synthetized to be used to learn if a different amino acid altered binding efficiency as reflected by cell activation. Paper 1 examined in detail two assay systems initially used, *in vivo* DTH skin reactivitiy and *in vitro* blastogenesis. It was found that distinct differences existed between the two assays. The major differences found were:

1. Only a limited range of antigen could sensitize rats sufficiently to yield positive *in vitro* blastogenesis to antigen. This limited dose range, permitting sensitization for *in vitro* responses, contrasted with the broad antigen dose range which sensitized for *in vivo* DTH skin challenge.
2. Strong DTH reactions were obtained with animals sensitized by either intraperitoneal injection or foot pad injection. In contrast, strong response with the *in vitro* assay of spleen cells worked only following intraperitoneal injection, but not

with foot pad injection of the sensitizing antigen.

3. When rat spleen cells were not pooled together and were tested for antigen induced blastogenesis, approximately one out of every four animals responded poorly or not at all. However, following *in vivo* DTH challenge, all animals produced strong, uniform reactions.

4. The kinetics of the appearance of maximum response were markedly different between the *in vivo* and *in vitro* assays. Maximum responses with DTH occurred 10-14 days after priming while maximum response with spleen cell blastogenesis occurred 19-20 days after priming.

Similar differences between DTH skin reactivity and *in vitro* blastogenesis were reported in guinea pigs immunized to *Staphlococcus aureus* antigens mixed in IFA. Myer-Bloch, et al. (1980) observed that the highest skin reactivity occurred on day 14, while maximum lymphocyte stimulation occurred 35 days after priming.

Paper 2 details distinct differences between the specificity of ABA-T and ABA-try antigens in the elicitaton of positive T-cell responses. These differences may be due to either a), the amino acid plays a crucial role in the recognition complementarity of the T-cell receptor or b), the amino acid may influence how Ia antigen (MHC) is altered during processing of antigen by macrophages prior to presentation of an antigen/MHC complex. Please refer to the diagram provided in the appendix for the following discussion.

Possibility b) takes into account the "altered self" theory. If an antigen/MHC complex is formed as stated by the theory, the first role of the amino acid may be to permit formation of an "altered-self" complex (i.e. be important for immunogencity), and second, influence the actual final structure of the complex, (i.e. determine what epitope is displayed), dependent upon the amino acid used by macrophages to create the complex. Possibility a) would not require the creation of an "altered self" complex. Rather, in addition to the ABA region, the amino acid region would also fit within the T-cell receptor paratope. This antigen T-cell receptor recognition would not require that the antigen be present on macrophages. However, interaction of some antigen with macrophage must occur. As suggested in the diagram, uptake of foreign antigen by macrophages initiates two events. First, Ia antigen is rearranged into a "new", altered form. A suggestion by Dr. Denis Burger (personal communication) is that Ia antigen is not expressed on macrophages until these cells interact with antigen. Second, uptake of antigen by macrophages stimulates the release of Interleukin-1, an essential factor in the development of antigen-induced T-cell responses (reviewed by Mizel, 1982). The expression of "altered" or <u>de novo</u> Ia antigen and the production of IL-1 would be the two required signals for a T helper cell subpopulation that specifically interacts with Ia antigen but does not require recognition of foreign antigen to function. The role of this T helper cell would be to produce a second required factor important

for antigen- induced T-cell response. This factor, Interleukin-2 (reviewed by Palacios, 1982) is important for maintaining mitogenesis during the immune response. IL-2, in our model, would be one of at least two signals required to initiate antigen specific, T-cell response initiating the appearance of IL-2 surface receptors. The second signal would be interaction of antigen with the T-cell receptor. When the two signals are received, the antigen specific T-cell (T effector cell), would release factors important for maintaining T helper cell production of IL-2, and lymphokines that would initiate immunological responses such as DTH. Thus, if this model were true, the role of the amino acid has for specificity would be at a later time period in the sequences of immune response. The amino acid may also be important for initiating macrophage response, i.e. expression of "altered Ia antigen" and production of IL-1, but would not show specificty of response at this level.

Other authors have provided some evidence that amino acids in the single amino acid conjugates are important for specificity. Thus, the results of Lawn & Leskowitz (1980), Jokipii, et al. (1975a & 1975b), and paper 2 here, suggests strongly that the amino acid uniquely contributes to the apparant specificty of T-cell response in the rat model. This study was not able to determine which of the two possibilities above may be true, but it seems likely that the T-cell receptor requires some physical contact with the amino acid for the recognition signal to be transmitted to the responding T-cell.

The model demonstrates that the amino acid present in the conjugate, is important to the specificity of T effector cells. However, the intriguing findings presented in paper 2, are that immune T-cells can differentiate between ABA-T and ABA-try; whereas, these antigen produce cross-reactive tolerance. The later effect maybe due to the induction of T suppressor cells which are specific for ABA only. We found that if either ABA-T or ABA-try was given at an early, immuno-incompetent age, it was not possible to immunize the animals with either antigen after maturation several weeks later. These results can be interpreted: 1) clones of cells could recognize the tolerizing amino acid conjugate were eliminated and a possible T suppressor population arose after the first injection that was specific for the ABA moiety alone. 2) Tolerance is less stringent than sensitization and all ABA recognizing cells, regardless of amino acid specificity, are "destroyed".

Some tentative evidence exists for T suppressor cells responsive to the ABA moiety presented in paper 2. Footpad immunized animals undergo poor blastognic responses to single amino acid antigens but produce strong response to ABA- proteins. This suggested to us that T suppressor cells responsive to the ABA group dominated the response to the single amino acid conjugates, whereas, the protein containing conjugate ABA-HGG has a strong interaction with macrophages and therefore a poor propensity to activte suppressor cells. We postulated that adding more ABA-T to wells already containing ABA-HGG would increase the inhibitory effects of

the T suppressor cells. This prediction was confirmed snce we found that the response to ABA- protein (ABA-39-HGG) was totally suppressed with the addition of increasing amounts of either ABA-T or ABA-try. These results could however occur through at least two mechanisms. 1) The receptors of T effector cells, specific to ABA-protein determinants, were blocked by the occupation of these receptors by the ABA group present in the single amino acid conjugates. 2) T suppressor cells, requiring only recogniton of the ABA moiety, were present and responded to either ABA-T or ABA-try by inhibiting other cells from responding to the ABA-protein. The second possibility was supported by the finding in another experiment looking for the presence of T suppresor cells specific for the ABA moiety. We chose a time when no antigen specific blastogenesis could be elicited. That is, ABA-T/CFA immune animals injected in the footpads, were sacrificed 10 days after sensitization. This protocol was found to be an extremely poor period of response for animals to challenge with ABA- conjugates <u>in vitro</u>. However, as mentioned before, animals primed with CFA tended to have high, nonspecific, background resonses. We postulated that T effector cells had not reached a functional state yet. We also expected ABA and PPD specific T suppressor cells to be present. Taking advantage of the non- specific, high background resonses of CFA primed animals, we found that the addition of increasing amounts of ABA-T, ABA-39-HGG or PPD blocked the non- specific background responses. These findings strengthen the likelyhood of antigen

specific T suppresor cells being present and that a clone of T suppresor cell could inhibit "background division" following recognition of the ABA moiety alone. The work of Lawn and Leskowitz (1980) using the same rat strain used in this study, provides additional evidence for the existance of ABA-specific T suppressor cells.

Though our work and the work of many others suggests that proteins or single amino acids are essential for immunogenicity of T-cell antigens, a recent intriguing report suggested otherwise. Ray and Ben-Sasson (1980) provided evidence that Hartley guinea pigs immunized with underivatized arsanilic acid (i.e. ABA free of any amino acid carrier), were capable of developing in vivo DTH skin reactions when challenged with ABA-Guinea pig albumin (GPA) and ABA-Ovalbumin (OA) did not react to DNP linked by an azo bond to GPA (DNPazo-GPA). In vitro, guinea pig lymph node cells (LNCs) primed with arsanilic acid responded to arsanilic acid, ABA-T, ABA-histidine, ABA-caproic acid, and three different ABA- protein molecules. However, animals primed to ABA-T responded strongly in vitro to only ABA-T challenge and responded mildly to either ABA-histidine or ABA-caproic. No response to arsanilic acid were detected with these cells. However, a note of caution in interpreting these results needs to be raised since, in contrast to in vivo results, in vitro responses of animals primed with arsanilic acid alone were less than one third the level of stimulation seen with in vitro responses following priming with amino acid conjugates

and challenged with the priming antigen. The authors suggests that arsanilic acid in guinea pigs, "without side chains (i.e. amino acid or protein conjugates) is immunogenic". To test this possibility in rats, we primed several groups of Sprague Dawley rats with ABA-T/IFA, ABA-T/CFA, arsanilic acid/CFA, arsanilic acid/IFA, or CFA alone. The results are shown in the the appendix. We found that positive skin reactions developed in both the ABA-T and the arsanilic acid primed animals, following ABA- protein challenge. These data and the data of Ray & Ben-Sasson could suggest that amino acids are not necessary for immunogenicity. However, little is known concerning arsanilic acid reactivity within the body. The possibility exists that this molecule may in some manner, become linked to "self" proteins and thereby providing the necessary carrier. One possibility of how this may occur was suggested by Dr. Jack Fellman (personal communication). Dr. Fellman suggested that the molecule is very similar in structure and chemical properties to the sulfonamides, which have been shown, in certain cases, to be hydroxylated by liver enzymes to form a molecule that spontaneously changes to a quinimine. Quinimines are strong alkylating agents that can then form covalent linkages with proteins. This could lead to arsanilic acid conjugation to self proteins via a different linkage than the diazonium bond normally used in synthesizing ABA-conjugated proteins and amino acids. However, it is unlikely that arsanilic acid <u>in vitro</u> could become covalently bound to proteins in culture making it difficult to understand how this compound would

activate cells *in vitro* if amino acids are truly required.

Significant to the above discussion is the finding by Ray and Ben-Sasson (1980) that ABA-T primed animals failed to respond *in vitro* to arsanilic acid challenge. This finding suggests that if ABA is linked to an tyrosine by a diazonium bond, a dominant clone of T-cells develops which recognizes both the arsonate and the amino acid moeities simultaneously. However, if arsanilic acid becomes linked to proteins or amino acids *in vivo*, in the manner suggested by Dr. Fellman, during sensitization, the ABA group would be linked to an amino acid directly onto the benzene ring of the benzenearsonate group (please refer to diagram in appendix relevant for this discussion). The primary structure of this linkage would contrast dramatically with the linkage of benzenearsonate group to an amino acid via a diazonium bond thus making it difficult to understand how ABA-T secondary stimulation of arsanilic acid primed animals could occur. Mild responses of arsnailic acid sensitized LNCs *in vitro* may indicate that undetermined "amplifying" effects are lacking with this cell population compared to ABA- amino acid sensitized cell populations. Also as mentioned before, DTH responses of animals primed with arsanilic acid are strong. Possibly the missing "amplifying" effects are present *in vivo* allowing for strong DTH but are missing *in vitro*.

Upon examination of the literature discussed in this thesis, a hierarchy of immuno- dominant determinants may be envisioned for ABA and its conjugates. The most immuno dominant determinants are

found on the highly immunogenic, foreign proteins. The presence of the ABA moiety has minimal effect on the overall dominant specificity of the induced immune response. This would be presumably due to the many clones of T-cells that can recognize the many different foreign determinants present. It would be likely that due to the "foreigness" of these proteins, processing and presentation would be very efficient. Next most dominant would be the neo- protein determinants created on poorly immungenic proteins such as self proteins as suggested by Ray and Ben-Sasson (1979). Fewer epitopes would be present on these proteins so fewer clones would be induced. The determinant created by conjugating ABA to single amino acids would be the third most dominant determinant in this hierarchy. This would be due to possibly only one or two clones of cells responding to the amino acid conjugate. Finally, ABA alone, missing amplification because of the lack of the amino acid, would be the weakest determinant in this hypothetical hierarchy. Limited responses *in vitro* could be due to either weaker interactions of arsanilic acid with T-cell receptors, or the poor processing of arsanilic acid by macrophages.

Use of *in vitro* analysis provides several distinct advantages, such as allowing subtle manipulaton of experimental conditons. Ideally, assays should measure "biologically relevant" functions. Due to the lack of a clear T-cell immune functon with *in vitro*

blastogenesis related in paper 1, we decided to explore the possibility of finding antigen- induced T-cell factors using a neutrophil assay recently developed in our laboratory (Bullock, et al., 1982). Using human PMNs in the original study, we adapted the neutrophil assay to be usable with rat PMNs. The application of both the aggregation assay, and a new assay for measuring nitro blue tetrazolium (NBT) for the study of rat PMNs, is described here. Rat neutrophils were obtained form Lewis female rats, by injecting a warm solution of oyster glycogen into the peritoneal cavity of the animals. Four hours later, the animals were sacrificed and the peritoneal cavity was injected with warm culture medium containing heparin. As shown by Wright's stain, the average number of neutrophils in this preparation was 77%. Cells were suspended in supplemented culture medium for overnight storage and on the following day were washed prior to use.

As found in paper 3, rat neutrophils were very sensitive to activation by phorbol myristate acetate (PMA) as indicated by cell aggregation. A similar result was found with human cells (Bullock, et al., 1982). Aggregation was blocked by the addition of either 2-deoxyglucose or idoacetamide, agents that block various levels of energy metabolism. It was found that rat neutrophils responded to LPS, but to a lesser extent then did to human neutrophils (Bullock, et al., 1982). Based on an original suggestion by Pick et al. (1981) using macrophages, it was decided to add NBT to activated neutrophil cultures. By simply changing the filter used for

aggregation studies from 410nm to 630nm, we found that we could readily detect NBT reduction. Thus, rat neutrophils, upon activation with PMA, could be assayed for either aggregation, NBT reduction, or aggregation followed by NBT reduction in sequence, using the same cell preparation. Rat neutrophils were found to be less efficient in reducing NBT as compared to human neutrophils (manuscript in preparation). This species difference could be due to: 1) different subpopulations of neutrophils (recently suggested by Klempner & Gallin, 1978), since the human neutrophils used from humans are obtained from the vasculature while the rat neutrophils used are obtained from the peritoneal cavity . 2) Differences in the serum components that influence neutrophil activity. 3) Intrinsic metabolic differences between the two animal species.

It is hoped that this assay will be useful in detecting T-cell lymphokine(s) that influenced neutrophil activity. Preliminary evidence presented here suggests such is the case. ABA-T immune rats, given a systemic challenge of the sensitizing antigen, released a putative lymphokine factor(s) detectable in the animal's serum four hours later. Serum from immune animals, but not serum from animals naive to the antigen, activated neutrophil aggregation in a dose response manner. Though the factor(s) that activates neutrophil aggregation has not been identified, work with human neutrophils stimulated with supernatants obtained from human T-hybridomas suggests that human neutrophils respond to T-cell factors by initiating aggregation and NBT reduction. The

preliminary data in humans also suggests that the induction of aggregation and NBT reduction are separatable events, since various T-hybridoma supernatants were found to induce either aggregation or NBT reduction, or both (data not shown). Though preliminary, the use of these neutrophil assays should provide useful, rapid, and quantitative methods for analysis of T-cell products that affect neutrophil activity.

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APPENDIX

Figure 1.

Female Sprague Dawley rats, 6 animals/group, were sensitized with either 100 mcg/animal ABA-T/IFA, ABA-T/CFA, p-arsanilic acid/IFA, p-arsanilic acid/CFA, or PBS/CFA only, given intraperitoneally. Twelve days later, all animals were skin tested with 100 mcg/site ABA_{39}HGG, ABA_{6}Oval, or HGG. Twenty-four hours later, skin reactions were measured (mm dia.). Displayed are the mean responses, +/- the SEM.

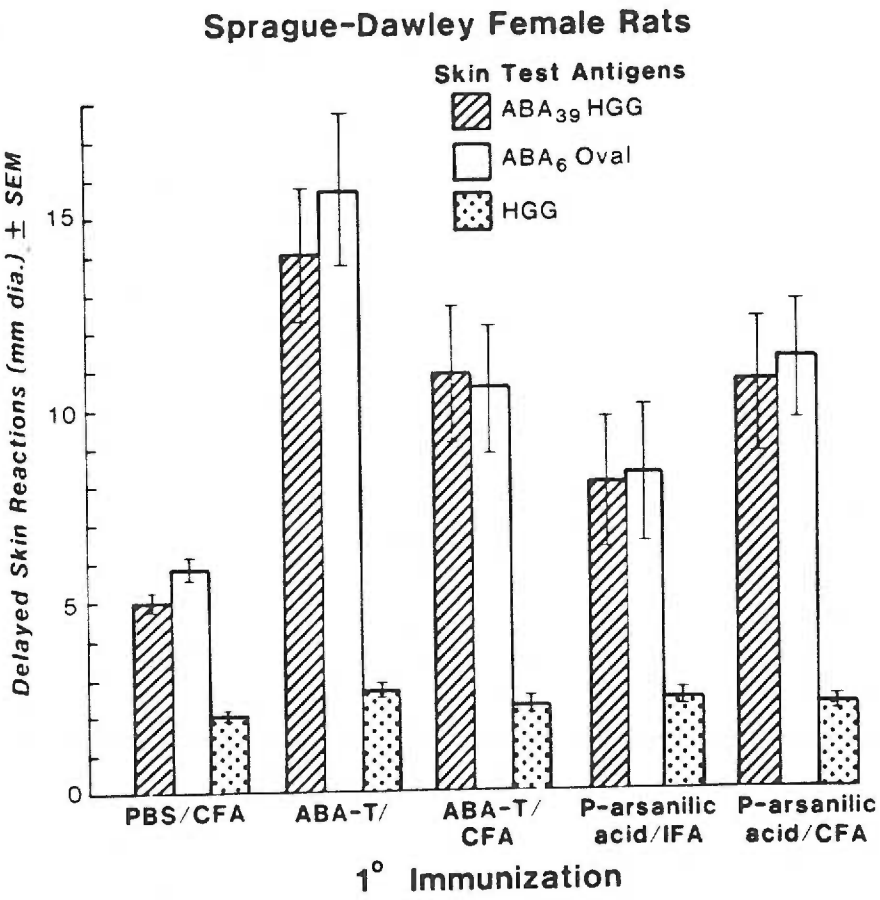

Sprague-Dawley Female Rats
Skin Test Antigens
ABA39 HGG
ABA6 Oval
HGG
Delayed Skin Reactions (mm dia.) ± SEM
0
5
10
15
PBS/CFA
ABA-T/
ABA-T/ CFA
P-arsanilic acid/IFA
P-arsanilic acid/CFA
1° Immunization

Figure 2.

Proposed model of events that take place during sensitization of animals to the small molecular weight antigen, ABA-T.

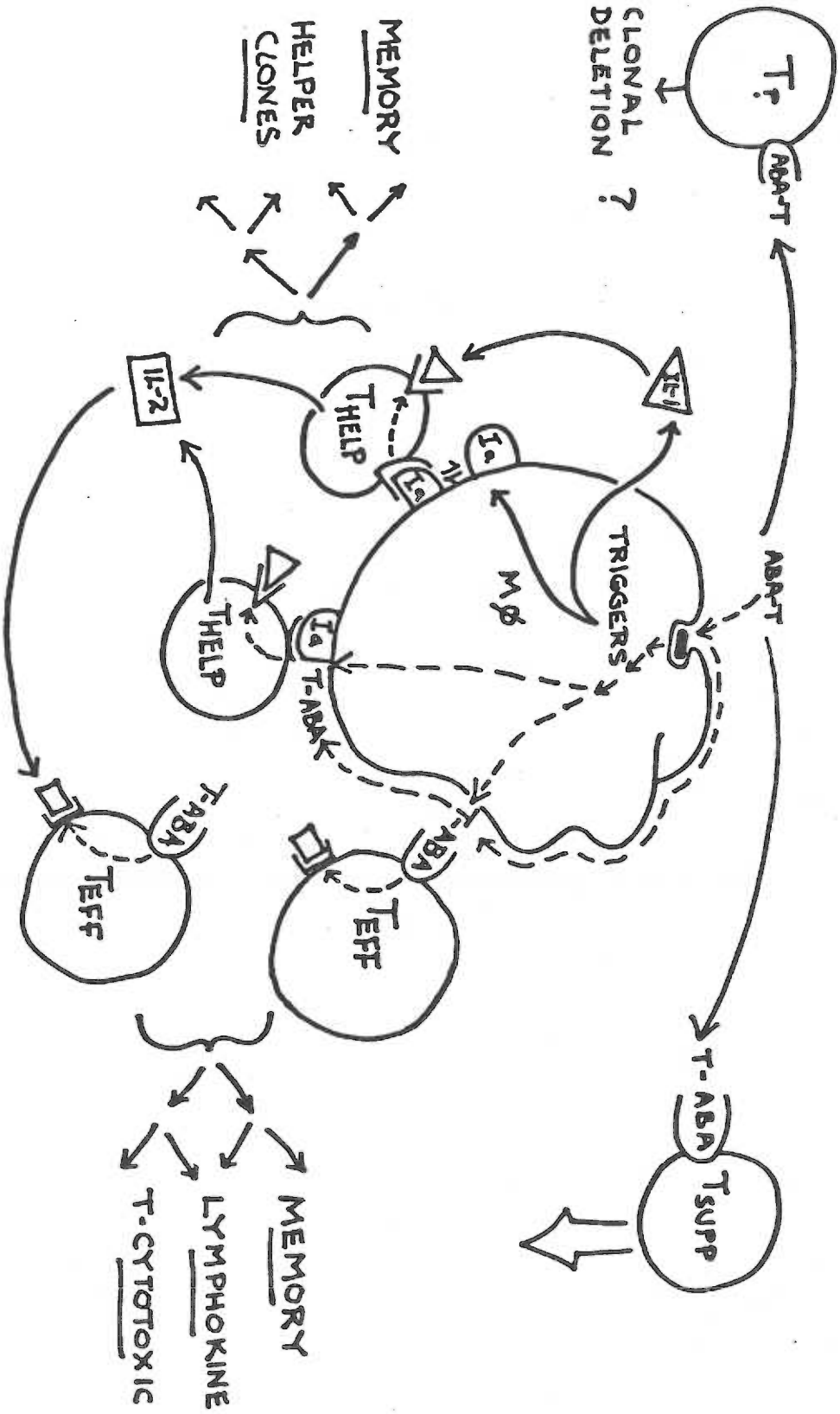
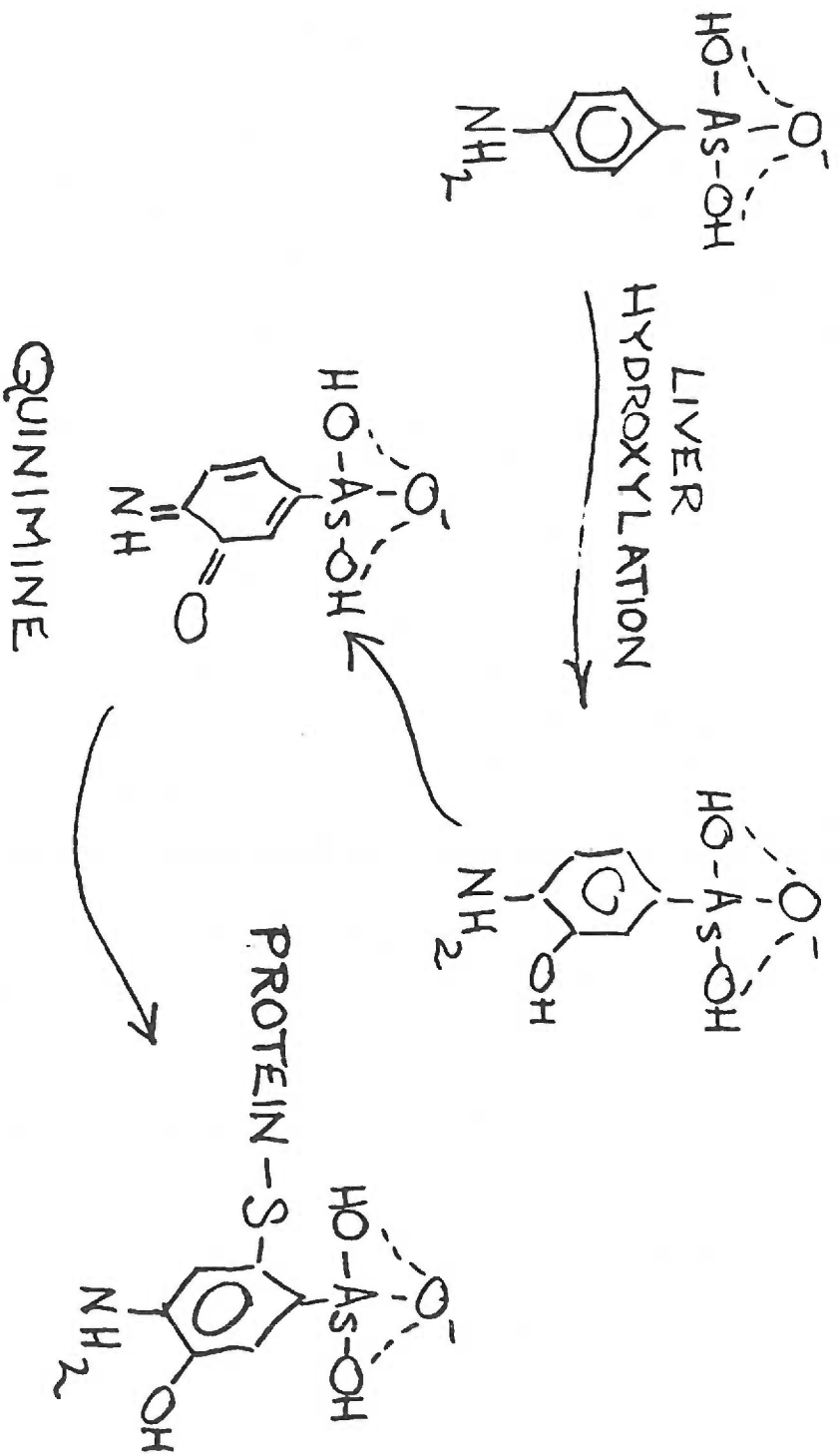

CLONAL DELETION ?
MEMORY
HELPER CLONES
IL-2
IL-1
MΦ
TRIGGERS
T-ABA
MEMORY
LYMPHOKINE
T-CYTOTOXIC

Figure 3.

Suggested reaction by Dr. Jack Fellman of p-arsanilic acid, converted into a reactive quinimine capable of binding to proteins in the body of an animal.


LIVER
HYDROXYLATION
QUINIMINE
PROTEIN-S-

THESIS SUMMARY

Single amino acids present in small molecular weight antigens, were studied for their role in the establishment of cellular immunity or tolerance. Two small molecular weight antigens were synthesized, p- azobenzenearsonate- N- chloroacetyl- L- tyrosine (ABA-T) and p- azobenzenearsonate- N- chloroacetyl- L- tryptophan (ABA-try), for this purpose. Initially, two assays were selected to assess the role amino acids play in tolerance and cellular immunity. It was found that the two assays, *in vivo* delayed type hypersensitivity (DTH) skin reaction and *in vitro* lymphocyte blastogenesis, displayed marked differences as for:

1. The optimum site for sensitization
2. The range of antigen dose yielding strong reactions.
3. The kinetics of the appearance of maximum response.

As for the role of amino acid present in ABA-T and ABA-try, we learmed that positive immune acitivity, both DTH skin reations and *in vitro* T-lymphocyte transformation, are ABA and amino acid specifie, i.e. little cross-reactivity was observed between the two antigens. In contrast, both *in vivo* tolerance and *in vitro* suppression were completely cross-reactive, preventing reactions regardless of the amino acid component used. The evidence presented here suggests that the fine specificity of cellular immunity to

ABA-T and ABA-try include recognition of the amino acid "carrier".

Because of the difficulties encountered with the in vitro blastogenesis assay, we decided to investigate whether or not a new assay could be used to examine antigen-induced release of T-cell factors that affect neutrophil function. We provide here suggested evidence that this indeed, may be done. Included is the description of the application of a rapid, quantitative assay measuring two neutrophil activities using the rat model.

ABBREVIATIONS

ABA : Azobenzenearsonate

ABA- : ABA conjugated to another molecule via a diazonium bond.

ABA-T : p- azobenzenearsonate- N- chloroacetyl- L- tyrosine

ABA-try : p- azobenzenearsonate- N- chloroacetyl- L- tryptophan

ABA-T/IFA : ABA-T emulsified 1:1 with Freund's incomplete adjuvant. A water into oil emulsion.

ABA-T/CFA : ABA-T emulsified 1:1 with Freund's complete adjuvant. A water into oil emulsion.

HGG : Human gamma globulin

Oval : Chicken egg ovalbumin

Ins : Insulin

PBS : 1x Sorensen's phosphate buffered saline.

IFA : Freund's incomplete adjuvant.

CFA : Freund' complete adjuvant.

PMA : Phorbol myristate acetate.

PMNs : Polymorphoneuclear cells (i.e. Neutrophils).

LPS : Bacterial Lipopolysaccharides

2-DOG : 2-deoxyglucose.

OD : Optical density.

SEM : The standard error of the means.

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And to one last individual, this thesis is sincerely dedicated to my mentor, Dr. Wesley W. Bullock. I can say no more than thank you for your help and being my friend.