THE MURINE IMMUNE RESPONSE TO BIFUNCTIONAL ANTIGENS

by

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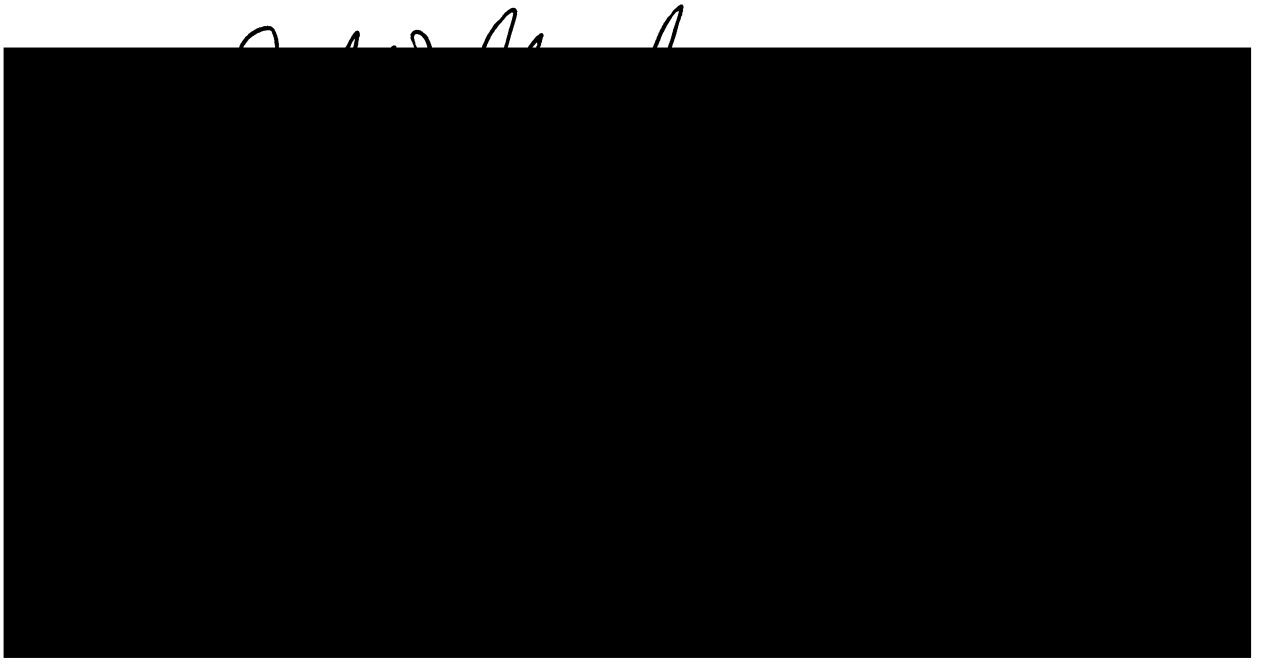

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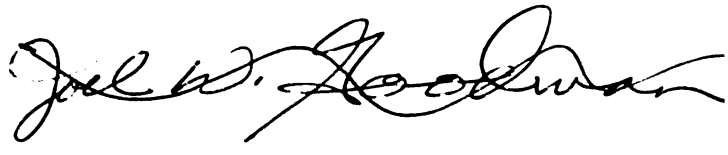
ABSTRACT

The object of this thesis has been to analyze and compare the murine immune responses in spleen and lymph nodes to bifunctional antigens (DNP-spacer-Tyr-ABA) comprised of only one haptenic (DNP) and one carrier (Tyr-ABA) epitope per molecule. Previously, the bifunctional antigens have been shown to be weak immunogens in mice, inducing only IgM plaque forming cells (PFC) in both primary and secondary responses. The main findings of this thesis are as follows: 1) Bifunctional antigens, when injected into the footpads (f.p.) and/or base of the tail, elicit an IgM and a much stronger IgG-PFC response in the local draining popliteal and inguinal lymph nodes, which contrasts with the response in the spleen. 2) Responsiveness to bifunctional antigens is genetically controlled, as is the case for many other synthetic antigens. Responsiveness to the bifunctional antigens, however, is not under Ir gene control. 3) The dose of the antigen does not affect the immunogenicity of the bifunctional antigens injected intraperitoneally (i.p.). The spatial relationship of the functional groups to each other is more critical than the molecular weights of these antigens. 4) Prior injection of bifunctional antigens intraperitoneally strongly suppresses (80-100%) a subsequent in situ lymph node anti-DNP response to similar antigens. 5) Suppression of the lymph node response is due to T suppressor cells induced in the spleen after i.p. priming. 6) These T suppressor cells actively transfer the

non-responsive state into f.p. primed recipients and a portion of them bind to the Tyr-ABA epitope. 7) The epitope of the bifunctional antigens responsible for the induction of suppression is Tyr-ABA. 8) T suppressor cells induced by Tyr-ABA partially suppress (30-40%) the anti-DNP response elicited by an antigen which contain no ABA functional groups. The same suppressor cells, however, do not suppress anti-SRBC (Sheep Red Blood Cells) or anti-BSA (Bovine Serum Albumin) responses. 9) A fraction of Balb/c anti-DNP PFC express the CRI_C idiotype, which is characteristic of anti-ABA antibodies in this strain. It is this fraction which is suppressed in Tyr-ABA i.p. primed mice.

These results emphasize the important role of different routes of immunization in generating different ratios of immunoregulatory cells, and, hence, different immune responses. Furthermore, they provide indirect evidence for a possible idiotypic connectance between anti-DNP and anti-ABA responses in Balb/c mice.



ABBREVIATIONS

ABA	azobenzenearsonate
CFA	complete Freund's adjuvant
CRI	major cross-reactive idiotype of A/J anti-ABA antibodies
CRI_C	major cross-reactive idiotype of Balb/c anti-ABA antibodies
dpi	days post immunization
DTH	delayed type hypersensitivity
DNP	2,4-dinitrophenyl
f.p.	footpad
GM	geometric mean
HBSS	Hanks' balanced salt solution
HEL	hen egg-white lysozyme
Id	idiotype
i.p.	intraperitoneal
KLH	keyhole limpet hemocyanin
Lys	lysine
MOPC-460	idiotype of Balb/c anti-DNP antibodies
OVA	ovalbumin
PBS	phosphate buffered saline
PC	phosphorylcholine
PFC	plaque forming cells
Pro	proline
sac	6-aminocaproyl
SE	standard error

SRBC	sheep red blood cells
T15	idiotype of the PC binding Balb/c myeloma protein
TNP	2,4,6-trinitrophenyl

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INTRODUCTION

Immunology has proven to be a complicated bio-science since it involves the study of several different sub-sets of lymphocytes and accessory cells and the network of their interactions.In order to understand how the immune system works,several approaches have been made to dissect it into simpler components to study each one of them in detail.

These studies can be categorized into two broad groups. The first group involves the limiting of the various classes of cells in an immune response.Some examples in this category are the use of cell sub-set specific antisera(1-3), various cell separation techniques(4-7), mitogens that selectively activate different classes of lymphocytes(8), immunopharmacological or physicochemical agents(9-10), and hybridoma or in vitro lymphocyte cloning techniques(11-12).

In the second group of studies, the interacting cells are left intact, but the number of clones of lymphocytes activated is restricted by using structurally simple antigens. These antigens can be divided into three sets:

1) Naturally occuring small proteins or their fragments,

2) Simple chemical groups (haptens) coupled to immunogenic carriers, and,

3) Synthetic peptides.

A couple of examples in the first set are, myoglobin(13), tobacco mosaic virus protein(14), lysozyme(15) and glucagon

(16). The second set, the use of haptens -pioneered by Landsteiner(17)- is currently the most widely used method. Simple chemical groups such as trinitrophenyl(TNP),dinitrophenyl(DNP), nitroiodophenyl(NIP), azobenzenearsonate(ABA), phosphorylcholine(PC), fluorescein(Fl) and several amino acids(18) are typically employed. The third type, synthetic peptide antigens, provide better tools because their structures can be tailored for the study of specific questions. Various examples are the multichain polymer of poly(L-tyrosyl,L-glutamyl)-poly-D,L-alanyl-poly-L-lysine, (T,G)-A-L(19-20) and its various analogs, a linear random terpolymer of L-glutamic acid-L-alanine-L-tyrosine, GAT(21,22); a copolymer of L-glutamic acid-L-tyrosine,GT(23); haptenated polymers of dinitrophenyl-L-lysine(24); bi- and trifunctional antigens of 2,4-dinitrophenyl-6-aminocaproyl-L-tyrosine-azobenzene-p-arsonate,DNP-sac-Tyr-ABA (25), and 2,4-dinitrophenyl-6-aminocaproyl-tyrosine-azobenzene-p-arsonate-nonaproline-tyrosine-azobenzene-p-arsonate, DNP-sac-Tyr-(ABA)-$(Pro)_9$-Tyr-ABA(26).

Bi- and trifunctional derivatives of the monofunctional antigen L-tyrosine-p-azobenzene-arsonate,Tyr-ABA have been extensively investigated in our laboratory.(Reviewed in 27,28). The prototype bifunctional antigen, DNP-sac-Tyr-ABA, is comprised of only one haptenic(DNP) and one carrier (Tyr-ABA) epitope per molecule and is capable of evoking both cellular and humoral immune responses in guinea pigs(25). The anti-DNP antibodies produced in response to DNP-sac-Tyr-ABA

are quite restricted in heterogeneity representing the products of probably fewer than three clones(29). In later studies aimed at looking at the relationship of spatial arrangements of functional groups to the immune response, haptenic and carrier epitopes were separated from each other by a rigid chain of poly-L-proline. Based on the humoral response of guinea pigs to various DNP-$(Pro)_X$-Tyr-ABA compounds DNP-$(Pro)_{22}$-Tyr-ABA is regarded as a representative antigen of this series(30). Bifunctional antigens were proven weak immunogens in several strains of mice. Given intraperitoneally, they evoked a weak IgM-plaque forming cell (PFC) response in the spleen(two to five fold over the background) without any IgG-PFC. Additional immunizations did not change the response profile. It was shown that the induction of a splenic IgG-PFC response was dependent on the addition of a second carrier epitope, thus making the molecule a trifunctional antigen (26).

In this context,it should be mentioned that the route of antigen injection affects the nature,strength and duration of the ensuing immune response.Antigens can be injected a number of ways:intraperitoneal,intradermal,intramuscular,intravenous,subcutaneous,oral etc.Particulate antigens such as red blood cells,bacteria,viruses or large polymers give very good antibody responses by most of the injection routes.However,protein antigens usually induce immunosuppression when they are injected intravenously,whereas other routes of immunization result in humoral and positive

cellular responses.Irrespective of the site of entry,antigens are distributed throughout the body via two circulatory pathways:the vascular and the lymphatic.Most of the antigens injected subcutaneously,e.g.,in the footpads or at the base of the tail,are carried by lymphatic vessels to the draining lymph nodes where they percolate in the node and then are passed to the blood circulation (Reviewed in 31).

The route by which antigen enters the body and gets transported to the lymphoid tissue also affects the localization of antibody producing cells.When antigen reaches the lymphoid tissue via the blood stream, it stimulates antibody production primarily in the spleen.Upon intravenous injection of bovine serum albumin (BSA) into rabbits,80-100 IgG-PFC per 10^6 cells were observed in the spleen,whereas mesenteric and peripheral lymph nodes contained less than 10 per 10^6 plaque forming cells (32).The same pattern is seen when antigen is injected intraperitoneally.Even with potent, complex antigens such as sheep red blood cells,relatively few PFC are found in the peripheral lymph nodes whereas the spleen contains large numbers of plaque forming cells (personal observations).Conversely,when antigen is injected subcutaneously,peripheral lymph nodes contain huge numbers of plaque forming cells (per 10^6 cells),whereas only a few plaques are observed later in the spleen (33).When antigen is given orally however, no plaques are detected in the spleen or in the peripheral lymph nodes (34).

The present study was aimed at inducing an IgG-PFC response to bifunctional antigens in mice by using a different route of immunization (other than i.p.) and investigating the consequences of intraperitoneal injection of bifunctional antigens. There are some examples in the literature indicating that injection of antigens subcutaneously at the base of the tail and into the hind footpads evokes stronger immune responses than i.p. injection. One of the best known examples is hen egg-white lysozyme (HEL). Mice of several strains, except those of H-2^b and H-2^s haplotype, give humoral and proliferative responses to HEL injected intraperitoneally (35,36). Furthermore, it has been shown that HEL induces suppressor T cells in nonresponder B10 (H-2^b) mice(37). However, the injection of HEL into the hind footpads induced excellent proliferative and PFC responses in the draining lymph nodes of the B10 mice, followed by high levels of serum antibodies (86,87).

Another extensively investigated example is the genetically controlled immune response to the random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}, GAT(41). Mice of H-2^p, H-2^q and H-2^s strains have been shown to be nonresponders(42) and to produce suppressor cells(43) and suppressive factors(44-46) upon intraperitoneal (i.p.) injection of GAT. However, by changing the dose (from 10 µgr to 100 µgr) and the route of injection from i.p. to footpad, Maurer(47) showed that the nonresponder H-2^s mice (others remained nonresponsive) did respond to GAT by T-cell prolifer-

ation and humoral antibodies. A similar situation exists in the response to L-glutamic acid-L-lysine-L-leucine, GLLeu : H-2^d mice previously considered to be marginal responders(48) gave very good responses after footpad injection of the antigen(49). The same phenomenon manifests itself in the sex-linked responses of nonresponder F_1 hybrid mice to TNP mouse serum albumin, TNP-MSA (50). Although F_1 female mice did not give a PFC response in the spleen after i.p. immunization, footpad injection resulted in a significant IgG-PFC response in the draining lymph nodes. Male mice remained nonresponsive in both instances.

In responses to transplantation antigens, comparative studies have been reported on different routes of immunization. It has been concluded that the strongest immune responses to transplantation antigens result from subcutaneous or intradermal injections(51,52). The popliteal lymph node enlargement test has proven sensitive enough for studying the responses to minor histocompatibility (H) antigens: Subcutaneous injections are done with allogeneic spleen cells into one footpad and syngeneic cells into the other, and the response is measured by weighing the draining lymph nodes. The test has been used for H-Y, one of the strongest of the minor H antigens (53), as well as for weaker antigens (54).

Response to the male specific H-Y antigen is one instance where changing the route of immunization has made a critical difference. The cytotoxic T cell, T_c, responses to this antigen were limited to H-2^b strain mice following i.p.

immunization and, according to this criterion, other strains were classified as nonresponders. However, changing the immunization protocol from i.p. to footpad injection showed that the putative nonresponder H-2^k(55), H-2^s and H-2^d(56)strains were able to mount a T_c cell response to H-Y. In yet another response to H-Y antigens involving the activation of helper T cells that would collaborate with B cells for antibody production, injection of H-Y antigen (as male spleen cells) at the base of the tail, followed by long-term in vitro culturing of the draining inguinal and para-aortic lymph node cells, resulted in a higly enriched T cell population specific for the H-Y antigen (57).

Differences in cellular organization of the spleen versus lymph nodes might give a clue about the differences found in immune responses in these organs after different routes of immunization. . If one looks at the general structure of lymphoid tissue, the spleen and lymph nodes show few differences. Both organs are compartmentalized into thymus-dependent (TD) and thymus-independent (TI) areas on the basis of histologic examination using reagents which distinguish between T and B lymphocytes (58,59). Lymphoid nodules and follicles in the white pulp of the spleen and the cortex region of lymph nodes constitute the TI areas, whereas the periarteriolar lymphoid sheath surrounding the artery in the white pulp of spleen and paracortical area of the nodes form the TD regions. The medullary cords of the nodes correspond to cords of red pulp in spleen. Macrophages and dendritic

reticular cells are dispersed along the reticular tissue that constitutes the stroma of both organs. However, the spleen has the marginal sinuses that surround the white pulp and also has the red pulp, both of which have no equivalent in the lymph nodes (60).

It is interesting that lymphocytes, which differ from other leukocytes by their low adhesive nature, readily bind to cultured fibroblast-like reticular dendritic cells obtained from peripheral lymph nodes or thymus(61). Since it has been shown that the fibrocellular reticulin fiber network constitutes the stroma of the lymph nodes(62), it is reasonable to believe that these reticular dendritic cells play an active role in cellular organization of the lymph nodes and are major sites of lymphocyte interaction .

One major difference between the spleen and lymph nodes is their connection to the rest of the system. In lymph nodes, lymphocytes and antigens enter the node either through the afferent lymphatics or from the arterial blood vessels and leave the node only via the efferent lymphatics. They do not return to the blood circulation in the nodes. In the efferent lymphatics 85% of the lymphocytes come from blood, 10% from afferent lymph and 5% are produced in the node de novo. About 25% of the lymphocytes in the arterial blood passing through the node enter the organ(63).

Lymphocytes enter the lymph nodes by adhering to and moving between the high endothelial venule (HEV) cells and then exit via the medullary regions and efferent

lymphatics(64). Lymphocytes enter the Peyer's patches by similar vessels; however, HEV cells are absent in the spleen. The importance of these cuboidal cells in the entry of lymphocytes into the nodes is implied by the fact that walls of these post-capillary veins are very thin in neonatally thymectomized or nude mice(65), and that they return to normal size after intravenous injection of lymphocytes(66).They are believed to play a very important role in the entry of lymphocytes into the nodes(67,68).

In contrast, lymphocytes and antigens enter the spleen via perifollicular sinuses with no special adherence to the endothelial cells of the sinuses and reach their destination by amoeboid movement through the meshwork of reticular cells. The entry is non-discriminative(69). The majority of lymphocytes leave the spleen via the venous blood vessels and a negligible part via the afferent lymphatics that flow from the spleen to the local lymph nodes(70).

Antigen is trapped and retained at two different locations in the lymph nodes. The first site is in the medulla and involves uptake by phagocytic macrophages. The bulk of the antigen is sequestered and degraded in lysosomal inclusions while some is retained in stable form in other cytoplasmic compartments.The second location is the lymphoid follicles in the cortex, where follicular dendritic cells (FDC) retain antigen extracellularly on their plasma membrane infoldings. The same kind of FDC is responsible for antigen presentation and immune induction in the spleen. Although

red pulp sinuses contain macrophages they have a minimal role in antigen presentation in the spleen (31).

Lymphocytes circulate throughout the lymphoid organs constantly. Both T and B cells take part in this traffic though B cells circulate much more slowly in comparison with peripheral T cells(71). In contrast to their non-discriminative circulation within the whole lymphoid system, their recirculation inside the lymphoid organs is very selective. T and B cells migrate mainly to the T and B dependent areas respectively, a phenomenon called as ecotaxis(72).Lymphatic cannulation experiments have provided valuable information about the recirculation of lymphocytes within the lymphoid tissue in rats(64,73,74),mice(75) and sheep(76-78).

An antigenic challenge markedly changes the lymphocyte recirculation. Studies of the lymph nodes have revealed that right after the challenge, blood supply to the node increases four-fold(79) due to enlargement of postcapillary venules(80), accompanied by a decrease in the efferent lymph output from the node. The latter is called "cell shutdown" (78,81) and is the result of complement activation and prostaglandin E_2 production(70,82,83).In addition, the antigen trapped in the lymphoid organs affects, by itself, the recirculation of lymphocytes. It has been shown that after the intravenous injection of antigen, the spleen becomes enriched in antigen specific lymphocytes whereas thoracic duct is depleted of such cells(84,85). The same is true for the entrapment of antigen-specific lymphocytes in the lymph nodes

after footpad challenge(86).The critical role of local antigen depots in recruiting specific lymphocytes to a particular site is best demonstrated in J.Thorbecke's experiments(267,268).After an antigenic challenge in both footpads, the lymph nodes from the left footpad,previously primed with the same antigen in the same side,gave higher PFC responses and transferred significantly more B cell memory responsiveness into recipient mice than the right side lymph nodes. Also,TNP primed memory cells,when injected intravenously into recipients which were immunized with TNP-KLH in the left and with ABA-KLH in the right footpad,were localized mainly in the left side lymph nodes.

It is believed that the follicular dendritic cells in lymph nodes play an important role in long term antigen entrapment as well as generation and retention of memory cells(269,87,88).Transfer of lymphoid tissue containing follicular dendritic cells from a primed donor to a non-immune recipient or to a culture medium without added antigen, results in induction of an antibody response called "spontaneous antibody response"(89,90).In a comparative study(90), spleen cells produced much less "spontaneous antibody response" than the draining lymph node cells,a result expected from the fact that lymph node tissues -draining an antigenic injection site- contain 20 times more antigen per tissue weight than the spleen(91). These accessory cells trapping the antigen locally, home in the draining lymph nodes and do not circulate(91,92). Antigen retention could

be quite long; in a rabbit system, "spontaneous antibody response" had been obtained from draining lymph node cells taken one year after antigenic challenge(93); interestingly, even after a year, non-draining lymph node cells did not give a response. Since retention of antigen on follicular dendritic cells does not happen without specific antibody(94,95), these antigen presenting cells most probably do not play a major role in the induction of an immune response.

Other antigen presenting cells, however, might play an important role in the induction and the outcome of an immune response. One such accessory cell is the Langerhans cell. These cells, although closely associated with epidermal cells, are highly mobile and rich in surface Ia-antigens. They travel in the afferent lymphatic vessels and upon reaching the regional lymph nodes, give rise to interdigitating cells. They are thought to ferry the antigen from the periphery by a membrane bound form to the local draining lymph nodes and present the antigen to the resident lymphocytes(96). The interesting results of M.Chase's experiments(97,98) can be interpreted with regard to the interaction of Langerhans cell with antigen and lymphocytes.It was found that excision of ears 3 to 6 hours after the intradermal injection of picryl chloride in the ear, prevented an otherwise good humoral and cellular immune response and resulted in an antigen-specific tolerant state. If, however, ears were excised later than 6 hours, no tolerance was observed. On the other hand, intravenous injection of the allergen at a dose equiv-

alent to the amount known to escape the local site after an intradermal injection via the blood vessels (in less than 3 hours), resulted in the same kind of tolerant state. An explanation of these results may be as follows: The immunogenicity of the antigen was achieved by the presentation of the antigen on Langerhans cells to the lymphocytes in the local lymph nodes, a process requiring more than 6 hours. Although the effect of the antigen escaping to the blood stream was tolerogenic, the antigenic stimulation in the nodes was the decisive factor to direct the immune system to a responsive state, giving rise to humoral and cellular responses.However, the excision of ears, 3 to 6 hours after the antigen injection did not permit the coupling of antigen to Langerhans cells and some of the antigen escaped in a short period of time to the blood circulation, thus causing an antigen-specific tolerance. (Detailed account of this subject will be presented in the Discussion Section.) The role of draining lymph nodes and Langerhans cells in contact sensitivity experiments are also marked by lack of response when the draining lymph nodes are removed either before or after the sensitization with 2,4-dinitrochlorobenzene(99,100), and when Langerhans cells are depleted by ultraviolet (UV) irradiation(101). The impairment of antigen presenting cell function by UV radiation has been reported by other investigators as well(102,103). Generation of suppressor T cells after antigen presenting cell depletion was also reported by Theze(104), Pierres(105), Ishizaka(106) and Pierce(107).

The present investigation, after establishing a local IgG-PFC response in the lymph nodes by f.p. immunization protocol, then focused on the effects of i.p. immunization. Some examples in the literature prompted us to look for suppressor cells induced in the spleen after an i.p. antigenic challenge. As mentioned before, in the HEL system, the $H\text{-}2^b$ mice gave proliferative and PFC responses in the draining lymph nodes after the f.p. challenge(38-40); but, on the other hand, these mice produced suppressor T cells in the spleen following i.p. immunization(37). Similarly,GAT evoked T-cell proliferation and humoral antibodies after the f.p. challenge in $H\text{-}2^s$ mice(47), but it induced suppressor cells in the spleen following i.p. immunization, again in the same $H\text{-}2^s$ strain of mice(43). The suppression of IgG,but not IgM,PFC for a hapten-carrier conjugate has been described by Tada(108). In that system, suppressor cells were generated by two sequential injections of carrier protein,KLH(Keyhole Limpet Hemocyanin) without adjuvant and the effects of suppression were assessed by deppression of the PFC response to DNP-KLH injected with adjuvant(109). It was observed that, in anti-DNP responses, only IgG-PFC was suppressed; suppressor T cells had little effect on IgM-PFC response. The precursors, effectors and target cells of the suppression have been well characterized in this system. Briefly,an antigen-binding T suppressor cell, generated by carrier priming,bore the Ly2+,I-J+ cell surface markers and produced an antigen specific soluble factor TsF which also bore the I-J marker and

V_H determinants. The action of this factor on an antigen-primed, radiation-sensitive and nylon-wool-adherent Ly1+,2,3+, I-J+ T cell, resulted in the generation of suppressor effector Ly2,3+,I-J+ T cells. This effector cell mediated its effects by producing an antigen-nonspecific factor which, in turn, acted on helper T cells across MHC and V_H differences (110-113). Tada had also reported another system in which only the IgE antibody response was suppressed in response to a DNP derivative of Ascaris suum extract(114). Similar consequential events in the generation of T cell mediated suppression have been described for GAT(44,46,270), GT(200,271), SRBC(272, 273), ABA(274,115) and NP(-16,117) systems. Although all of the cells involved in the generation of suppression were not described in each system, sufficient similarities among them prompted Germain (118) to propose a common suppressor pathway for all of these antigen induced suppression systems. In the consensus suppressor pathway, it is postulated that the first exposure of antigen causes an antigen-specific, idiotype bearing and cyclophosphamide(CY) sensitive pre-Ts_1 cell (Ly1+ or Ly1+,2,3+ and I-J+) to differentiate to a mature Ts_1 cell and secrete a factor, TsF_1. This factor is antigen specific and has the I-J marker and the idiotype; furthermore, the factor is not MHC or $V_H$restricted in activity. Upon a second antigen exposure, this factor induces a pre-Ts_2 cell(Ly1+,2,3+, I-J+, I-C+ not CY sensitive) to become a Ts_2 cell(Ly2,3+, I-J+, I-C+). This idiotype-specific or antigen-specific Ts_2 cell, then secretes a factor, TsF_2(I-J+, I-C+) which is either anti-idiotypic

and V_H restricted or antigen specific and MHC restricted in activity. TsF_2 triggers a third cell, Ts_3 (Ly1+,2,3+, I-J+, idiotype bearing, CY or radiation sensitive), to secrete a nonspecific factor TsF_3 to mediate the suppression. It is also possible for Ts_2 or Ts_3 to act directly on target T cells through antigen-bridging or idiotype-antiidiotype mechanisms.

Idiotypes were first described on myelomas(119), on human(120) and rabbit(121) antibodies and on B cells(122-124). Idiotypic determinants were later found on helper(125, 126), suppressor(127,128), cytotoxic(129) and antigen binding T cells(130,131) as well. Certain antigens induce antibodies, the great majority of which bear a common or cross-reactive idiotype. T15, X24, CRI, A5A and J558 are examples of such idiotypes, expressed on anti-phosphorylcholine(132), anti-galactan(133), anti-arsonate(134), anti-streptococcal group A carbohydrate(135) and anti-α 1-3 dextran(136) antibodies, respectively.

CRI was initially defined on serum anti-ABA antibodies of A/J mice, hyperimmunized with ABA-KLH(134). CRI positive anti-ABA antibodies were in the range of 35-50% in individual antisera(137,138). Studies of monoclonal anti-ABA antibodies generated by somatic cell fusion have shown that CRI positive A/J anti-ABA antibodies are a family of non-identical but closely related molecules(139-141). CRI idiotype is also expressed on ABA-specific suppressor T cells(128,142) and ABA-specific T_{DTH} (delayed type hypersensitivity) cells(143).

As more monoclonal anti-ABA antibodies were generated, it became apparent that there were actually two groups of idiotypes-major and minor- associated with A/J anti-ABA responses. The minor idiotype defined by Nisonoff(144) constitutes 5-10% of anti-ABA antibodies, the rest belonging to CRI_A-major idiotype. A minor idiotype in A/J mice was also reported by Marshak-Rothstein(145-146) and designated as Id^{36-60}. Both groups have also shown that at least part of the A/J CRI minor idiotypic family is related to a major idiotype, CRI_C, expressed on Balb/c anti-ABA antibodies(147-148).

The idiotype expressed on the TNP-binding Balb/c myeloma protein, MOPC-460, has been found on anti-TNP or anti-DNP antibodies induced by the injection of T independent (149) or T dependent(152) conjugates of TNP (or DNP). The MOPC-460 idiotype, M-460 Id,has been probed by anti-idiotypic reagents prepared in rabbits (150,152), in syngeneic mice (149), or by monoclonal anti-idiotypes generated by somatic cell fusion (153). Comparison of M-460 Id with M-315 Id (which is expressed on the closely related TNP-binding syngeneic myeloma protein, MOPC-315) by the rabbit anti-idiotypic reagent has shown that each protein has two sets idiotypic determinants, one public idiotype which is shared by both proteins and one private idiotype different for each one (151). Several mouse strains immunized with DNP-BGG expressed more public than private M-460 idiotype; on the other hand, private M-315 Id was not detectable(153). Expression of high levels of M-460 Id in the serum after DNP-OVA challenge was

determined by genes governing heavy chain variable and κ-light chain variable regions(154). This expression was transient and peaked early in the response. The same transient expression was also found at the plaque forming cell level: M-460 positive plaques quickly diminished after secondary antigenic challenge, even when the number of anti-DNP plaques was still high. Nevertheless, 52-91% of the plaques were idiotype positive during the early phase of the response(155). M-460 Id was also found in the nonimmune serum of several strains of mice. M-460 Id positive molecules,however, were not DNP-binding and it was postulated that these molecules were specific for some environmental pathogen(156). On the other hand, analysis of M-460 Id by syngeneic monoclonal anti-idiotypic reagents(153) distinguished the presence of at least two idiotypic determinants (or idiotopes) which were not cross-reactive. Only one group of these idiotypic determinants were recurrently expressed in Balb/c anti-DNP antibodies after antigenic challenge with DNP-OVA(157).

There are several reports(158-163) indicating that idiotypes can also be shared by antibodies of different specificity. For the antigens hen eggwhite lysozyme(159) and sperm whale myoglobin(163), the antibodies that shared a common idiotype were specific for different epitopes of the antigens. There are also some examples where antigenic specificities of the antibodies involved were not known. Sharing of M-460 Id by DNP-binding and nonbinding antibodies(156, its authors believe that idiotype bearing DNP-nonbinding protein

is an immunoglobulin) was mentioned in the preceding paragraph. Similar results have been reported for anti-ABA antibodies also: two different idiotypic families , CRI^{AD8} and CRI^{5Ci}, were found on both ABA-binding and ABA-nonbinding antibodies (162,164). Like M-460 Id, CRI^{AD8} -but not CRI^{5Ci}- was also found in the serum of unprimed mice (162). Another example of a major idiotypic family expressed in the serum of normal mice is the T15 idiotype (165). In the T15 system, endogenous antibodies did bind to the same epitope, phosphoryl choline, as the antibodies induced after antigen priming, whereas in both the M-460 Id and the CRI^{AD8} cases, the specificities of endogenous idiotype-bearing proteins were not determined. It was argued that biologically the most meaningful explanation of these results would be that these are antibodies generated in response to an endogenous stimulus, such as enteric flora or food antigens. The fact that M-460 Id levels are indeed greatly reduced in germ free mice (156), favors the idea of a microbial origin.

Existance of these idiotypes -also called "regulatory idiotypes" (166)- might be an important element in the interpretation of the immune system as a web of different circuits of lymphocytes -specific for different antigens- which are, nevertheless, connected and hence subject to regulation by common immunoregulatory cells by virtue of their common idiotypes. Such idiotypes have been depicted in Jerne's network theory of the immune system (167). In the network system, immunoglobulin molecules and cell receptors

on a lymphocyte recognizing an epitope of an antigen, display both a set of paratopes -antibody combining site, and a set of idiotypes -antigenic determinants displayed on variable regions of the antibodies. The basic elements of the network system are as follows: The paratope (p_1) of a set p_1i_1, in addition to recognizing the epitope (E_1) of an antigen, also recognizes the idiotype (i_2) of another set, p_2i_2. Since i_2 and E_1 are recognized by the same paratope, they are predicted to be similar, and, accordingly, the p_2i_2 set is called the "internal image" (of the epitope). On the other hand, the idiotype of the responding set, i_1, is recognized by the paratope, p_3, of an anti-idiotypic set p_3i_3. The paratope, p_3, also recognizes the same idiotype, i_1, which is expressed on an "unspecific parallel set", p_xi_1. The paratope p_x recognizes an epitope different than E_1, hence is "unspecific" for E_1. The idiotype i_1 in the network system corresponds to a "regulatory idiotype" which is shared on antibodies having specificities for different epitopes.

The subject of the present investigation, the bifunctional antigens, promise to be an interesting case for a study looking at the immunoregulation of the responses to these antigens, especially in Balb/c mice. Its epitopes, DNP and Tyr-ABA are widely used in Immunology, and hence a vast amount of information is available. Furthermore, the idiotypes, M-460 Id and CRI_C, associated with anti-DNP and anti-ABA responses, have been described in Balb/c mice, as mentioned in earlier paragraphs. There is no a priori rea-

son to assume that any two epitopes would be connected by some regulatory idiotype. However, if a major idiotype as found to be shared between lymphocytes specific for ABA and DNP, then the effects of the regulation of the immune response to bifunctional antigens would be very pronounced. For example, if a suppressive response were to be induced, then the suppression would be very dominant (90-100% reduced responses), because, anti-idiotypic suppressor cells would act on both classes of lymphocytes activated in response to carrier(ABA) and haptenic(DNP) determinants. Nearly all DNP-specific B cell clones in connection with the shared idiotype would be suppressed. In the course of the present investigation, it was found that the suppression induced in response to bifunctional antigens was indeed very dominant, and preliminary data hinted the possibility of an idiotypic connectance between anti-DNP and anti-ABA responses.

The regulatory idiotypes would help the immune system to economize in the number of immunoregulatory cells it employs for ordering the interactions of activated lymphocytes. The same set of immunoregulatory cells would be used for different antigenic responses if the lymphocytes specific for these different antigens share an idiotype.The examples in the preceding paragraphs ensure the reality of regulatory idiotypes, but more examples are needed to validate the universality of this concept.

MATERIALS AND METHODS

1. ANTIGENS

The synthesis of monofunctional L-tyrosine-p-azobenzene-p-arsonate (Tyr-ABA), bifunctional N-2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine-p-azobenzene-p-arsonate (DNP-sac-Tyr-ABA) or N-2,4-dinitrophenyl-(proline)$_{22}$-L-tyrosine-p-azobenzene-p-arsonate (DNP-Pro$_{22}$-Tyr-ABA), and trifunctional N-2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine-p-azobenzene-p-arsonate-(proline)$_9$-L-tyrosine-p-azobenzene-p-arsonate (DNP-sac-Tyr-ABA-Pro$_9$-Tyr-ABA) has been previously reported (25,30,168). One of the bifunctional antigens, DNP-Pro$_{22}$-Tyr-ABA was synthesized by the solid-phase technique (169). Briefly, N-tert-butyloxy-carbonyl-O-2,6-dichlorobenzyl-L-tyrosine (BOC-OBzCl$_2$-Tyr) was esterified to the chloromethylated styrene polymer, and tri-proline peptide derivatives (N-BOC-Pro$_3$-OH) were added to the polymer by symmetrical anhydride fragment coupling (170). After 21 prolines were coupled to the polymer, DNP-Pro-OH was added as the N-terminal residue and the peptide was cleaved from the polymer by liquid hydrogen fluoride. The cleaved peptide was chromotographed on Sephadex LH-20, and then coupled with diazotized arsanilic acid. The compound DNP-Pro$_{22}$-Tyr-ABA was finally purified on Sephadex LH-20. The other bifunctional antigen DNP-sac-Tyr-ABA was synthesized by coupling the DNP-sac with tyrosine methyl ester. The ester was removed by alkaline hydrolysis and the peptide was coupled with diazotized

arsanilic acid. The end product was purified on Sephadex G-15 and LH-20 in 0.1 N ammonia. Tyr-ABA was prepared by coupling N-protected (N-tert-butyloxycarbonyl) tyrosine to diazotized arsanilic acid, followed by purification on Sephadex G-15. N-protecting group was removed with formic acid. The other antigens DNP-OVA, ABA-BSA, TNP-LPS and DNP-Ficoll were kindly provided by G.K.Lewis. Bovine serum albumin (BSA) was purchased from Calbiochem,San Diego, California., and N-ε-DNP-L-Lysine from Sigma,St.Louis,Missouri.

2. ANIMALS

Female Balb/c, C57Bl/6 and A/J mice, 6-8 weeks of age, were obtained from A.Griffin, University of California, Berkeley. Female SJL, SWR, DBA/1 and CBA/J mice, 6-8 weeks of age, were purchased from The Jackson Laboratory, Bar Harbor, Maine.

(CBA/N X Balb/c) F_1 mice of both sexes were bred in our breeding colony and were used at 7-10 weeks of age. CBA/N parents were provided by G.K.Lewis.

3. IMMUNIZATIONS

All antigens were dissolved in phosphate buffered saline (PBS) at 2 mg/ml (except in the dose-kinetics experiment) and emulsified in an equal volume of complete Difco Freunds adjuvant H37Ra. A total volume of 0.1 ml of the emulsion was injected either intraperitoneally or at the base of the tail and into the hind footpads.

4. PLAQUE FORMING CELL ASSAY

A modification of Jerne's Hemolytic plaque assay (171) was employed as follows: Single cell suspensions were prepared from popliteal and inguinal (or para-tymic in Experiment 2) lymph nodes or from spleen, using a 100 mesh cell screen (Cell-Rad Inc., Lebanon, PA.). Cells were washed in Hank's balanced salt solution, HBSS (Grand Island Biological Co., Grand Island, N.Y.). Plaquing aliquots for each petri dish (13- X 100-mm, 1029; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard. Calif.) contained 0.5 ml of 0.5% Bacto agar (Difco Laboratories, Detroit, Mich.), 290 µgr of DEAE-dextran (Sigma Chemical Co., St.Louis, Mo.), 100 λ of appropriately diluted cell suspension, 50 λ of indicator SRBC, 50 λ of 1:5 diluted guinea pig complement (Grand Island Biological Company, Grand Island, N.Y.) and 100 λ of developing reagents for indirect plaques. Developing reagents consisted of 50 λ of goat anti-mouse IgM (used at a final dilution of 1:128) for inhibition of IgM plaques and 50 λ of rabbit anti-mouse IgG (at a final dilution of 1:1448) for developing of IgG plaques. Both antisera were purchased from Cappell Laboratories Inc., Cochranville, Pa., and optimum dilutions for the assay were determined by a titration experiment using SRBC primed spleen cells. The IgG subclass specific plaques were developed by rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 antisera (Miles Laboratories, Elkhart,IN.). All of the antisera were used at a final dilution of 1:128 into the plaquing medium and titrations were made by plaquing the

following ABA specific cell lines: 36-65 (IgG1), AB2-104.3X (IgG2a), AB2-143.2X (IgG2b), and AB2-213.2 (IgG3). The cell line 36-65 was provided by A.Marshak-Rothstein and the rest by G.K.Lewis. Each plaquing sample was vortexed thoroughly in a small test tube and was spread onto the petri dish as a thin layer. Petri dishes were incubated in a humid plastic box at 37°C until the plaques were developed (2-4 hours).

For anti-DNP plaque forming cells (PFC), TNP-SRBC was used as indicator cell. It has been shown that TNP-SRBC (172), and DNP-SRBC(173) detected the same number of anti-DNP PFC. 30 mg of TNBS in 7 ml of cacodylic buffer was reacted with 1 ml of packed SRBC, and TNP-SRBC cells were washed with phosphate buffered saline (PBS) extensively. ABA-SRBC were prepared by imidoester coupling as described previously (174). For anti-BSA assay, BSA was coupled to SRBC by the method of Golub(175) using following amounts of chemicals: 700 mg of BSA and 500 mg of 1-ethyl-3-(3-dimethylaminopropyl) -carbodiimide-HCl (ECDI) were reacted per 0.5 cc of packed SRBC. Both BSA and ECDI were purchased from Sigma Chemical Co., St.Louis,MO.

5. CELL TRANSFERS AND α-Thy-1+C' TREATMENT

Donor mice were primed intraperitoneally with antigen and 2 weeks later their spleens were removed. Single cell suspensions were made and $1x10^7$ cells in a total volume of 0.2 ml were injected intravenously into the heparin treated (10 units/mouse, injected 1 hr. before the cell transfer)

recipients. One or two days before the cell transfer, recipient mice were primed with antigen in the hind footpads.

For the depletion of T cells, spleen cells were resuspended in the supernate of the anti-Thy-1 antibody secreting cell line, HO-13-4 (maintained in our laboratory), at a concentration of $1.5x10^7$ cells/ml. After a 40 minute incubation on ice, cells were spun down and brought back into the original volume in a 10% solution of agarose absorbed (176) guinea pig complement. Incubation was continued in a 37°C waterbath for another 40 minutes, and the cells were then washed 3 times with HBSS. This procedure usually kills 30-35% of the splenic cells.

Effectiveness of the α-Thy-1+C' treatment was assessed by comparing the SRBC response -a T cell dependent response- of the T cell depleted spleen cells with control spleen cells in an adoptive cell transfer experiment. Recipient mice received 700 rads whole body X-irradiation (Cesium source, U.C.San Francisco, Radiology Dept.), one day before the cell transfer and were placed on chlorinated acid water. $1x10^7$ spleen cells were injected intravenously and mice were then challenged with SRBC ($6x10^8$ SRBC in 0.5 ml of HBSS/mouse) intraperitoneally. 6 days later, spleens were assayed for anti-SRBC PFC. The following results were obtained from spleens populated with α-Thy-1+C' treated cells: 208±55 IgM-PFC/spleen and 0±0 IgG-PFC/spleen. Control spleens produced 5,608±500 IgM-PFC/spleen and 8,486±2,665 IgG-PFC/spleen. The results were found to be satisfactory.

6. PANNING ON ANTIGEN COATED PLATES

Separation of antigen-specific T cells from spleen cells was achieved by adsorbing them on antigen coated plates according to a modified method of Lewis(177). B cells and plastic surface adherent cells were removed from the spleen cell suspension by two successive panning on anti-mouse immunoglobulin coated plates and flasks. 5-6 ml of the protein solution at a concentration of 1 mg/ml was used per plate or flask. The first panning was done on flasks (Corning 150 cm^2 tissue culture flask #25210) at 5°C for one hour; and, the second panning on plates (VWR 150x15 mm dispoable petri dishes #25384-139) at 23-24°C again for one hour. The non-adherent cells (25% of the original cell input) were collected and washed twice with HBSS. Half of the cells were adsorbed on ABA-BSA coated plates and the other half on DNP-GPA (DNP-guinea pig albumin) coated plates. 5-6 ml of either ABA-BSA or DNP-GPA solution, 1 mg/ml, was used per plate coating. Adsorption on antigen coated plates were done at 23-24°C for one hour. In all adsorption procedures, cell concentration was $1.5x10^7$ cells/ml. Antigen non-adherent cells were collected by a pipette without disturbing the surface, and antigen-binding cells were recovered by vigorous washing of the plates. Both cell populations were extensively washed with HBSS before transfer.

7. PREPARATION OF ANTI-IDIOTYPIC REAGENTS

Rabbit anti-MOPC-460 sera were produced according to the method of Rosenstein et al (150) by two injections of rabbits with affinity purified MOPC-460 protein (1 mg of protein in CFA per rabbit). MOPC-460 myeloma cells were carried in Balb/c mice by weekly ascites fluid transfers. The antiserum was passed over columns of Sepharose 4B coupled with MOPC-315 protein, and anti-idiotypic antibodies specific for public idiotype shared between MOPC-460 and MOPC-315 proteins were recovered from the column with hapten (DNP-glycine) elution. In ELISA assay, this anti-MOPC-460 idiotypic reagent bound to MOPC-460, MOPC-315 and 36-60 (a generous gift of Ann Marshak-Rothstein), but not to TEPC-15 coated plates. Binding of the reagent to MOPC-460 plates was inhibitable by MOPC-460, MOPC-315 and 36-60 proteins, but not by TEPC-15 protein.

Rabbit anti-CRI_c sera were produced by the method of Brown et al (147) by repeated immunization of rabbits with affinity-purified Balb/c anti-ABA antibodies from hyperimmune ascites (This anti-CRI_c sera were prepared by a joint work with J.D.Conger.). Rabbit antiserum was first passed over BDF_1 and A/J Ig coupled Sepharose 4-B columns and then was rendered Id specific by passage over columns coupled with normal Balb/c Ig [$(NH_4)_2SO_4$-precipitated proteins from Balb/c hyperimmune ascites induced by CFA alone].

Monoclonal anti-idiotypic reagent specific for MOPC-460 idiotype was a generous gift from P.A.Cazenave.

8. DATA PRESENTATION AND STATISTICS

Groups of 4 mice were used in each experiment. Results for each group were presented as GM± SE (Geometric Mean ± Standard Error). Plaque forming cell values per 10^6 cells were recorded for each mice separately. Geometric means and standard errors were calculated by the following formulae:

$$\log GM = \frac{\sum_{i=1}^{n} \log x_i}{n}$$

$$SE = \frac{SD}{\sqrt{n}} \quad , \quad SD = \left(\frac{\Sigma (x-\bar{x})^2}{n-1} \right)^{\frac{1}{2}}$$

In suppression experiments, percent suppression was calculated using geometric means of PFC /10^6 values of experimental (suppressed) and control (nonsuppressed) groups as follows:

$$\% \text{ suppression} = \frac{PFC_{control} - PFC_{exp}}{PFC_{control}} \times 100$$

Significance of suppression was assessed by the Student's t test for paired samples(178).A probability value of < 0.01 was considered significant and values less than 0.001 very significant.

RESULTS

1. POPLITEAL AND INGUINAL LYMPH NODE RESPONSES TO BIFUNCTIONAL ANTIGENS

Injection of the bifunctional antigen, DNP-Pro_{22}-Tyr-ABA (100 µgr in CFA), subcutaneously at the base of the tail and into the hind footpads led to a significant IgG-PFC response in the draining popliteal and inguinal lymph nodes of Balb/c mice (Fig.1). The response peaked at 14-15 days post immunization (dpi), and values for anti-DNP IgM-PFC and IgG-PFC were 10-15 and 110-120 PFC per 10^6 viable lymphocytes, respectively. CFA controls gave practically no response: 0-2 IgM- and 0 IgG-PFC/10^6. The PFC responses in the lymph nodes were rather short-lived; by 16 days the number of IgM and IgG anti-DNP plaques dropped to 6 and 59 PFC/10^6, respectively. Microscopic examination of the lymph node cells, however, showed that nodes were still populated with lymphoblasts and the plasma-cell-like lymphocytes. Anti-DNP plaque assay of the spleen indicated that activated cells in the lymph nodes were able to circulate; IgG-PFC values rose from 0 to 51, 182, 358, 478 PFC/spleen following 10, 13, 14, 16, and 18 days after footpad immunization, respectively. There was no significant change in IgM-PFC values: Background levels of 500-3000 IgM-PFC/spleen remained unchanged.

2. SPLENIC RESPONSES TO VARIOUS BIFUNCTIONAL ANTIGENS

Previous experiments with bi- and tri-functional

antigens were carried out using strain A/J mice. To determine if the observed IgG-PFC response was due to the use of a different strain of mice (Balb/c) rather than the footpad injection, several bifunctional antigens were injected intraperitoneally and the spleens were assayed for anti-DNP IgM- and IgG-PFC. Results (Fig.2) were very similar to previously reported ones in the A/J strain (26): No IgG-PFC were observed and IgM-PFC were only 2 to 4 fold over the CFA controls. Furthermore, there were no significant differences between the results obtained with DNP-sac-Tyr-ABA, DNP-Pro_{10}-Tyr-ABA, DNP-Pro_{22}-Tyr-ABA and DNP-Pro_{31}-Tyr-ABA.

The popliteal, inguinal and mesenteric lymph nodes of i.p. primed animals were also assayed for anti-DNP plaques, and neither IgM- nor IgG-PFC were found. Nevertheless, a previously published work (179) justified further search for anti-DNP PFC in other lymphoid organs. It was reported in(179) that,following i.p. injection of a protein antigen,the highest numbers of the PFC were to be found in the parathymic lymph nodes. Accordingly, groups of mice were injected with either DNP-sac-Tyr-ABA or DNP-Pro_{22}-Tyr-ABA and their parathymic lymph nodes were assayed for anti-DNP PFC. Results were not different from those in spleen: Numbers of direct plaques were in the range of 10-50 IgM-PFC/10^6. Although IgM-PFC values were similar for both antigens, DNP-Pro_{22}-Tyr-ABA -but not DNP-sac-Tyr-ABA- induced a small (20 PFC/10^6) IgG-PFC response. Since this response was not high, the subject was not pursued further.

Recruitment of helper cells -induced by i.p. priming - into the spleen following a secondary i.p. challenge was tried for the purpose of inducing an IgG-PFC response in spleen.First,mice were primed with DNP-PRO_{22}-Tyr-ABA in the footpads, then either 10 or 30 days later animals were challenged i.p. with DNP-sac-Tyr-ABA. Spleens were assayed for direct and indirect plaques 6, 9, and 11 days later.The range of IgM-PFC was very similar to the primary response: 40-50 IgM-PFC/10^6.Again,no IgG-PFC were observed.

3. DOSE-KINETICS EXPERIMENT WITH DNP-sac-Tyr-ABA

We had previously been using a dose of 100 µgr of bifunctional antigen per mouse for both intraperitoneal and subcutanous footpad injections.This decision was based on the previous results (26) with bifunctional antigens in mice and,incidentally, it coincided with the number of molecules of antigen used per gram body weight of guinea pigs (1 µmole/guinea pig) that was shown (30) to give an optimum response. Nevertheless,to make sure that we were not missing an IgG-PFC response in the spleen after i.p. immunization due to a non-optimal antigen dosage,a dose-kinetics experiment was done.A broad range of antigen doses were tested: 500 µgr, 25 µgr, and 0.5 µgr per mouse and the response was followed from day 4 to day 13 post immunization (Fig.3).The results were similar to the ones obtained with 100 µgr of antigen.We did not observe any IgG-PFC and there were no significant differences among the different doses used.Therefore,we concluded that

the failure to detect indirect (IgG) plaques in the spleen was not due to low-zone or high-zone tolerance.

4. LYMPH NODE RESPONSES TO DNP-sac-Tyr-ABA, DNP-PRO_{22}-Tyr-ABA AND DNP-PRO_{16}-sac-PRO_{15}-Tyr-ABA

Since there was no significant difference in splenic responses to various bifunctional antigens injected intraperitoneally(Fig.2), it was of interest to determine if similar results would be obtained by footpad injection. One difference among the three bifunctional antigens used in this experiment was the physical distance between the haptenic(DNP) and the carrier(Tyr-ABA) determinants. In DNP-sac-Tyr-ABA (D-s-R), the flexible sac chain had an extended length of about 8 A° whereas the rigid left-handed helix of the Pro_{31} spacer in DNP-Pro_{31}-Tyr-ABA (D-P_{31}-R) had a length of about 97 A° (based on the axial translation of 3.12 A° per proline residue in the polyproline helix). In DNP-Pro_{16}-sac-PRO_{15}-Tyr-ABA (D-P_{16}-s-P_{15}-R), the two functional groups can come closer together by bending of the molecule due to the flexible -sac- chain inserted in the middle. Results in Table I indicated that the distance between the two determinants influences the indirect plaque responses quantitatively. In ascending order, D-s-R, D-P_{31}-R and D-P_{16}-s-P_{15}-R gave 23, 86, and 231 IgG-PFC/10^6. These results combined with those of Experiment 1 where D-P_{22}-R evoked 120 IgG-PFC/10^6, implied that mice gave best responses to the bifunctional antigens when the haptenic and carrier determinants were at an optimum distance from each

other: This distance should be between 8 A° (D-s-R) and 97 A° (D-P_{31}-R). The result -231 IgG-PFC/10^6- obtained with D-P_{16}-s-P_{15}-R, further strengthened this conclusion: Although this antigen had 31 proline residues between the two functional determinants, it may have acted like D-P_{22}-R owing to the flexibility of the sac spacer. Since the synthesis of D-P_{22}-R was easier than D-P_{31}-R or D-P_{16}-s-P_{15}-R the decision was made to use DNP-Pro_{22}-Tyr-ABA for footpad injections in further experiments. It was also decided to continue the use of DNP-sac-Tyr-ABA for i.p. injection, because its preparation did not require solid-phase peptide synthesis and there were no differences in splenic PFC for different bifunctional antigens.

5. GENETIC CONTROL OF THE LYMPH NODE RESPONSE TO DNP-Pro_{22}-Tyr-ABA

In addition to the physical distance between the DNP and Tyr-ABA groups in DNP-sac-Tyr-ABA and DNP-Pro_{22}-Tyr-ABA, the other difference between these two molecules is the existence of proline residues in DNP-Pro_{22}-Tyr-ABA. We probed the question of proline help by using three strains of mice that were shown by others (180,181) to have distinctive responses to poly-proline. Mice of the H-2^s haplotype were the only responders to GL-Pro and hence to proline, because GL was not immunogenic in all strains tested (180). Also, SJL (H-2^s) mice were high responders and DBA/1 (H-2^q), SWR (H-2^q) were low responders

to (T,G)-Pro-L (181). We assumed that if the better lymph node response to DNP-Pro_{22}-Tyr-ABA were largely due to proline residues, we should expect very high responses in SJL mice and comparatively low ones in DBA/1 and SWR mice. However, the results of genetic control experiment (Table II) did not confirm our assumption: SJL mice turned out to be low responders to the bifunctional antigen. DBA/1 and SWR mice were practically non-responders. Since the SJL's high response potential towards proline did not overcome its low responsiveness to DNP-Pro_{22}-Tyr-ABA, we concluded that proline residues in this molecule were not the major cause of high responsiveness in the lymph nodes of Balb/c mice. The results with SJL, DBA/1 and SWR mice prompted us to check the other strains for a possible association between the responsiveness to DNP-Pro_{22}-Tyr-ABA and H-2 haplotype (Table II). Our results identified CBA/J ($H\text{-}2^k$), C57B1/6 ($H\text{-}2^b$), and A/J ($H\text{-}2^a$) as nonresponders, low responders and high responders, respectively. The discrepancy in responsive states of CBA/J and A/J mice tends to rule out the possibility that responsiveness to bifunctional antigens is under histocompatibility (H) -linked Ir gene control because CBA/J and A/J are both H-2 I^k. H-linked immune response genes have been mapped to the I region of the murine H-2 complex (182).

6. A) LYMPH NODE RESPONSES OF CBA/N x Balb/c HYBRIDS TO BIFUNCTIONAL ANTIGENS

In this experiment, we wished to check the contribution of Lyb 3,5(+) B cells to the IgG-PFC lymph node response to bifunctional antigens. Lyb 3(+) B cells constitute 40-50% and 95-100% of the total B cells present in the spleen (183) and in the lymph nodes(184), respectively. The Lyb 3 cell surface marker is absent on B cells from CBA/N mutant mice and acts as a receptor on mature B cells for T-cell-derived helper factors (185). CBA/N mice have a sex-linked recessive xid gene defect (comprehensive reviews of the subject can be found in refs. 186-190) which is expressed only in male offsprings of a cross between a CBA/N female and any normal male. We expected that if the higher ratio of Lyb 3(+) B cells in lymph nodes were the major cause of IgG-PFC responses, then, CBA/N ♀ x Balb/c ♂ (CBF_1) males should give little or no response while their female siblings should show normal IgG-PFC in the popliteal and inguinal lymph nodes following footpad injection of DNP-Pro_{22}-Tyr-ABA. The results, presented in Figure 4, complied to our expectation. CBF_1 females and males gave cumulative PFC values of 17±13 and 1±1 IgG-PFC per 10^6 cells, respectively (days 14-18 post immunization). The Student t-test performed on data corresponded to a p value of less than 0.001 which indicated that the difference between females and males was highly significant. Another interesting part of the experiment was that responsiveness to the bifunctional antigen was inherited in a

co-dominant Mendelian fashion. A cross between a high responder and a non-responder produced intermediate-responder hybrids.

B) RESPONSE OF [Balb/c ♀ x CBA/N ♂] F_1 MICE TO TI-1, TI-2 AND TD ANTIGENS

In experiment 6-A, some differences were observed (Fig.4) between male and female offsprings of the control cross, Balb/c ♀ x CBA/N ♂, BCF_1. Male BCF_1 mice gave higher responses (15-60 IgG-PFC/10^6 cells) than female BCF_1 mice (5-22 IgG-PFC/10^6 cells). Although both sexes of BCF_1 were phenotypically normal, female BCF_1 mice had nevertheless one defective X chromosome. Since only a small number of BCF_1 mice were used for that study,the experiment was repeated,this time using one TI-1 antigen, TNP-LPS, one TI-2 antigen, DNP-Ficoll and one TD antigen, DNP-OVA. To avoid polyclonal activation, only 10 µgr of TNP-LPS and DNP-Ficoll were used per mouse. DNP-OVA was used at the dose of 100 µgr/mouse and all antigens were injected into the footpads. 4 to 14 days later, both spleens and lymph nodes were assayed for anti-DNP plaques. Results presented in Table III showed no significant differences between males and females for any of the three antigens. First of all, male and female BCF_1 mice responded to DNP-Ficoll equally well. If the xid gene in female BCF_1 mice were to influence the response, then, this effect would have had to be very pronounced in DNP-Ficoll response; the xid defect influences mostly the TI-2

antigen responses (186-190). Secondly, the PFC responses to TNP-LPS and DNP-OVA were similar in both sexes. We did not know whether the differences between male and female BCF_1 responses, observed in Experiment A,were due to the bifunctional antigen used or were due to the use of too few mice.

Although mice were injected in the footpads, their spleens gave anti-DNP PFC as well, indicating that some antigen had reached the spleen to evoke a response. The draining lymph nodes responded as expected, except for TNP-LPS. Very curiously, no anti-DNP plaques were observed in the lymph nodes in this group, although the response in the spleens of the same animals were normal. Some possible explanations of this last point will be considered in the Discussion Section.

7. ISOTYPIC PROFILE OF LYMPH NODE RESPONSES

The antibody response to T-independent types I and II (TI-1,TI-2) antigens and to T-dependent (TD) antigens shows a characteristic isotype pattern for each antigen(191). In this experiment, we investigated the isotypes of the anti-DNP antibodies induced in response to bi- and trifunctional antigens injected into the hind footpads. To enumerate each isotype, we developed the IgG plaques with isotype-specific antisera at a dilution which was determined to be optimal. Titration and specificity tests were done with several hybridomas secreting antibodies of different isotypes.

The results (Fig.5) were typical of the TD antigens: the great majority of the indirect plaques (77-84%) were of the IgG1 type which was followed by intermediate (12-21%) values of IgG2a+b, and a negligible part (2-3%) was IgG3 type.

One other characteristic of TD antigens is that, after a second challenge, they evoke a higher response -especially in the IgG compartment- and the peak of the response is reached earlier. In a previous work (26), no secondary response was found (same level of IgM-PFC and no IgG-PFC) in the spleen of mice which were primed and boosted intraperitoneally with bifunctional antigens. We tried priming and boosting, both in the footpads. One month after the mice were primed with DNP-Pro_{22}-Tyr-ABA, they were challenged with the same antigen. 8,10 and 13 days after the challenge, the lymph nodes were assayed. IgG-PFC values (per 10^6 cells) for days 8,10 and 13 were 250,20 and 13, respectively. The data showed that the secondary IgG-PFC values (250 IgG-PFC/10^6) were higher than the primary ones (110 IgG-PFC/10^6). Although from the above data we could not tell the exact day of the peak in the secondary response, it was apparently reached before the peak of the primary response -day 14-; because, the response in day 8 was much higher than the responses in days 10 and 13. These results indicated that bifunctional antigens evoked a TD-antigen-like secondary response.

8. EFFECT OF CYCLOPHOSPHAMIDE (CY) TREATMENT ON SPLENIC RESPONSE

Cyclophosphamide (CY) has been widely used as an immunomodulator. Depending on the dose and the time of administration, it has been shown to suppress or enhance immune responses (192). Enhancement of humoral or cellular responses at low doses of the drug (20 mg/kg body weight, 1 day before the antigenic challenge) has been attributed to its effect on suppressor cells(193-195). At intermediate doses (100-200 mg/kg body weight) it starts to affect B cells as well, but the enhancement of cellular responses still persist (196-198). Nevertheless, examples can be found where administration of CY at intermediate doses (100-200 mg/kg body weight), 2-3 days before the antigenic challenge, leads to increased humoral responses (199-200). Based on these observations, we wished to see if the administration of CY would induce an IgG-PFC response to bifunctional antigens in the spleen. Two different doses of CY were used; 20 mg/kg body weight and 100 mg/kg body weight. The results presented in Table 4 indicate that CY was not effective in overcoming the non-responsive state of the spleen after an i.p. immunization with DNP-sac-Tyr-ABA or DNP-Pro_{22}-Tyr-ABA.

9. EFFECT OF DNP-sac-Tyr-ABA i.p. IMMUNIZATION ON FOOTPAD CHALLENGE WITH DNP-Pro_{22}-Tyr-ABA

Bifunctional antigens gave an IgG-PFC response in lymph nodes after footpad injection, but none in the spleen

following i.p. immunization. It seemed possible that the absence of indirect plaques in the spleen was the result of the action of suppressor cells induced in that organ in response to the antigen. The fact that CY treatment was not effective in abrogating these putative suppressor cells did not deter us from pursuing the matter, because it has also been shown that CY is not effective for all kinds of suppressor cells (118,192). In this experiment, we looked for a direct demonstration of suppression induction by i.p. immunization. To do this we injected several groups of mice intraperitoneally with DNP-sac-Tyr-ABA, and 1,2,3 and 14 weeks later we challenged the mice with DNP-Pro_{22}-Tyr-ABA in the hind footpads and assayed the draining lymph nodes for anti-DNP IgG-PFC. Results presented in Table V show that a prior i.p. immunization suppressed a subsequent in situ IgG-PFC response to the bifunctional antigens in the lymph nodes. The suppression could be shown one, two or three weeks after the primary challenge, but it was absent after fourteen weeks, clearly showing that the mice were not rendered permanently tolerant. From then on, we employed a two week time interval for generation of the suppressed state. Another conclusion derived from this experiment was that the suppression was antigen specific. It did not suppress the response to bovine serum albumin (BSA).

10. TRANSFER OF SUPPRESSION BY SPLEEN CELLS

After demonstrating the non-responsiveness induced by i.p.immunization,we wished to know if this non-responsiveness was due to an active suppression mechanism, i.e., whether the non-responsive state could be transferred with spleen cells from suppressed donors to recipients which were challenged in the footpads with bifunctional antigen. We intravenously transferred 10^8 or 10^7 cells from donors which were immunized i.p. with DNP-sac-Tyr-ABA or CFA 2 weeks before, into syngeneic recipients. The recipients were challenged with the bifunctional DNP-Pro_{22}-Tyr-ABA in the hind footpads 1 day before the cell transfer. 5,8 and 14 days later, lymph nodes were assayed for direct and indirect anti-DNP plaque forming cells. Results presented in Figure 6 clearly demonstrate the existence of an active suppressive mechanism. Percent suppression reached the level of 90% for both groups. With 10^8 and 10^7 CFA primed control spleen cells transferred into syngeneic recipients, we observed a phenomenon similar to that described by Celada (201,202) as the "syngeneic transfer barrier". Transfer of higher number of cells (10^8/mouse) gave a smaller response (137 IgG-PFC as opposed to 215 IgG-PFC observed with 10^7 cell transfer) and the peak of the response shifted to an earlier day - day 8 instead of day 11 for 10^7 cell transfer . One way to overcome this syngeneic barrier was to irradiate the recipients at a low dose before cell transfer. However, when we irradiated the recipients at a dose of 200 rads, the popliteal and

inguinal lymph nodes became too small to work with. We therefore altered the protocol such that the recipients were immunized before cell transfer in order to overcome feedback inhibition on the part of the recipients against the transfer of primed cells.

11. T CELL NATURE AND ANTIGEN SPECIFICITY OF THE SUPPRESSOR CELLS

In this experiment, we adressed the question of the nature of the suppressor cells which transferred the non-responsiveness and also whether they would behave in an antigen-specific fashion in their action like they do in in situ responses. The experimental protocol and results are presented in Figure 7. Spleen cells from DNP-sac-Tyr-ABA primed donors actively transferred suppression, reaching levels of 89-97% suppression. The same cells did not suppress the anti-SRBC (Sheep Red Blood Cells) when transferred to SRBC primed recipients.

Anti Thy-1 plus complement (α-θ+C') treatment of spleen cells from DNP-sac-Tyr-ABA i.p. immunized mice failed to suppress the response of DNP-Pro_{22}-Tyr-ABA primed recipients. Thus, this experiment showed that suppressor cells induced in response to i.p. priming with bifunctional antigens were antigen-specicific T suppressor cells.

12. ADSORPTION OF T SUPPRESSOR CELLS ON ANTIGEN COATED PLATES

After demonstrating the antigenic specificity of T suppressor cells, both in situ and in cell transfer experiments, we were interested to know whether these cells would bind to the antigen. To address this question, we performed the following antigen adsorption experiment: B cells and plastic adhering cells were removed from spleen cells taken from suppressed mice. After two stage panning on anti-mouse Ig antibody coated plastic plates at 4°C (to prevent the capping of B cells) and at 23°C (to remove any remaining B cells or plastic adhering cells),the non-adherent cells were divided into two groups. One group was panned on ABA-BSA and the other on DNP-GPA coated plastic dishes at 23°C. Non-adherent cells and adherent cells -removed by vigorous pipetting with cold medium- were transferred separately into footpad primed recipients. Results are presented in Table VI. B cells and plastic adherent spleen cells recovered from anti-mouse Ig coated plates were also transferred as positive controls and gave responses very similar to CFA control values (260 and 180 IgG-PFC,respectively). Suppressor T cells did not bind to DNP-coated plates selectively, the amount of suppression observed for adherent and non-adherent cells was almost the same: 86 and 85 %,respectively. In contrast,panning on ABA-coated plates resulted in an enriched suppressor T cell population. Recipients of ABA-adherent and non-adherent cells showed the following IgG-PFC values: 8 and 59 per 10^6 cells, corresponding to 97 and 77 % suppression,respectively. The

different IgG-PFC values of the adherent and non-adherent groups corresponded to a p value less than 0.005 indicating a significant difference between the groups. It is important to point out that panning on ABA plates did not separate all the suppressor T cells from the rest of the population.

13. EPITOPE OF THE BIFUNCTIONAL ANTIGEN RESPONSIBLE FOR THE INDUCTION OF SUPPRESSION

Previously published results from our laboratory had indicated that the Tyr-ABA epitope of the bifunctional antigen acted as the carrier determinant recognized by T cells, while the DNP epitope acted as the hapten for B cell recognition. Likewise, in the present study we never found any anti-ABA PFC in the footpad primed mice, and in the panning experiment we observed that at least a fraction of suppressor T cells recognized and bound to the ABA group specifically. We were interested in seeing whether intraperitoneal injection of Tyr-ABA alone would induce a suppression similar to that seen with the bifunctional antigen. In this experiment, we also included DNP-Lys i.p. injected mice as a separate control group. Results (Fig.8) showed no difference between the suppression induced either by DNP-sac-Tyr-ABA or Tyr-ABA i.p. injection. In this particular experiment, both regimens resulted in almost 100 percent suppression of the anti-DNP PFC response in the lymph nodes. Conversely, DNP-Lys i.p. injection did not lead to suppression; in Figure 8, it can be seen that DNP-Lys injection

seemed to evoke an even better response than the CFA controls. However, Student's t test applied to paired groups of data (belonging to 7,9,11,13,15 and 18 days post immunization), corresponded to p values greater than 0.5 for all groups, indicating that the difference between cfa(i.p.) and DNP-Lys (i.p.)groups was not significant.

14. EFFECT OF SUPPRESSION ON ANTI-DNP AND ANTI-ABA RESPONSES

Thus far we have analyzed the effects of the i.p. injection of mono or bifunctional antigens on the lymph node response to f.p. challenge with bifunctional antigens. As the results indicated, i.p. immunization led to a dominant suppression. The observed 90-100% suppression is higly striking. It seemed interesting to test the effects of Tyr-ABA epitope-induced T suppressor cells on anti-DNP and anti-ABA responses evoked by conventional antigens, such as DNP-OVA and ABA-BSA. The magnitude and kinetics of the PFC response to f.p. injection of DNP-OVA (Fig.9-A) and ABA-BSA (Fig.9-B) was different: 32,300 versus 180 IgG-PFC per 10^6 cells on day 8 versus day 11 post immunization for DNP-OVA and ABA-BSA antigens, respectively. The suppression of the anti-ABA response (with prior i.p. sensitization with DNP-sac-Tyr-ABA) was 19, 40 and 11 percent for 11, 13 and 19 day responses. Considering the variation among individual mouse responses (p value was between 0.4 and 0.5), the suppression of anti-ABA responses was not great.

In three different experiments, we observed a 40-55

percent suppression of anti-DNP response to DNP-OVA or DNP-BSA. Even with transferred suppressor T cells we saw a 53% suppression (in the panning experiment) with a p value of 0.005 . In all these instances, we have induced the suppressor T cells with an i.p. injection of DNP-sac-Tyr-ABA. In the present experiment we included a third group: suppressor cells induced with Tyr-ABA alone (First and second groups were CFA controls and DNP-sac-Tyr-ABA groups, respectively). We were interested in knowing whether suppressor cells induced by this monofunctional antigen would also suppress anti-DNP responses to an antigen which contained no ABA groups. The results presented in Figure 9-A and Table VII-A showed that Tyr-ABA did indeed suppress anti-DNP responses to DNP-OVA. DNP-sac-Tyr-ABA or Tyr-ABA i.p. injected groups gave similar IgM and IgG anti-DNP PFC values and also similar degrees of suppression: 30-80 IgM and 500-18,000 IgG-PFC per 10^6 cells for both groups, and 30-50 % versus 30-40 % suppression of IgM and IgG responses, respectively.

15. A PRELIMINARY IDIOTYPIC ANALYSIS OF THE RESPONSE

The results obtained from the third group in Experiment was indeed very interesting: T suppressor cells induced in response to the ABA group were able to suppress a response to an unrelated group, DNP. Since the antibodies specific for one group do not cross-react with the other, the possibility existed that the antibodies specific for ABA and DNP groups might be sharing some idiotypic determinant(s).

As a result, immunoregulatory lymphocytes recognizing these determinants could regulate both responses, even when they were generated only by one of these determinants. Accordingly, a rabbit anti-MOPC-460 anti-idiotypic reagent was prepared and tested to enumerate M-460 Id(+) anti-DNP plaques, but this reagent did not inhibit any of the anti-DNP plaques. Next, we tested an anti-CRI^{c} anti-idiotypic serum. Results in Table VII-B showed that 16-76% of the anti-ABA PFC induced in response to ABA-BSA were CRI^{c} positive on the basis of inhibition with this reagent. A previous i.p. immunization with DNP-sac-Tyr-ABA did not change the percentage of CRI^{c}(+) anti-ABA plaques. Results obtained in the inhibition of anti-DNP plaques proved to be interesting. First of all, anti-CRI^{c} which distinguishes the idiotypes expressed on Balb/c anti-ABA antibodies, also recognized some determinants on Balb/c anti-DNP antibodies produced in response to DNP-OVA. 22-55% of the anti-DNP plaques in the control mice were inhibited by anti-CRI^{c}. The other interesting result was the absence of CRI^{c}(+) plaques in the suppressed groups. When mice were injected i.p. with DNP-sac-Tyr-ABA or Tyr-ABA and then challenged in the footpads with DNP-OVA, all of the anti-DNP plaques were then CRI^{c}(-). These last results seemed to confirm our hypothesis that anti-ABA anti-DNP antibodies shared some idiotypic determinants, here designated CRI^{c-460}. Suppressor cells (anti-idiotypic ones) induced by Tyr-ABA were thus able to regulate anti-DNP responses by suppressing CRI^{c-460} idiotype-bearing DNP-specific lymphocytes.

We then received a monoclonal anti-MOPC-460 reagent. This reagent, unlike rabbit antiserum, did inhibit anti-DNP plaques produced in response to bifunctional antigens. 39-51% of anti-DNP plaques were M-460 Id(+). The results are shown in Table VII-C. Mice suppressed with an i.p. injection of DNP-sac-Tyr-ABA or Tyr-ABA did not produce enough anti-DNP plaques after footpad challenge with the bifunctional antigen to assay for idiotype positive plaques.

DISCUSSION

The object of this thesis has been to analyze and compare the murine immune responses in spleen and lymph nodes to bifunctional antigens (DNP-spacer-Try-ABA) comprised of only one haptenic (DNP) and one carrier (Tyr-ABA) epitope per molecule. The main findings are as follows:

1) Bifunctional antigens, when injected into the footpads and/or base of the tail, elicit an IgM and a much stronger IgG-PFC response in the local draining lymph nodes than in the spleen.
2) Responsiveness to bifunctional antigens is genetically controlled, as is the case for many other synthetic antigens.
3) Prior injection of bifunctional antigens intraperitoneally strongly suppresses (80-100%) a subsequent in situ lymph node response to similar antigens.
4) Suppression of the lymph node response is due to T suppressor cells induced in the spleen after i.p. priming.
5) These T suppressor cells actively transfer the non-responsive state into f.p. primed recipients and a portion of them binds to the Tyr-ABA epitope.
6) The epitope of the bifunctional antigens responsible for the induction of suppression is

Tyr-ABA.

7) T suppressor cells induced by Tyr-ABA partially suppress (30-40%) the anti-DNP response elicited by an antigen which contains no ABA functional groups.

These results emphasize the important role of different routes of immunization in generating different ratios of immunoregulatory cells and, hence, different immune responses. Furthermore, they provide indirect evidence for an idiotypic connectance between anti-DNP and anti-ABA responses in Balb/c mice.

Employing the i.p. method of immunization, bifunctional antigens have been shown to be weak immunogens eliciting only weak IgM and no IgG-PFC responses in mice, even after repeated boosting (26). Nevertheless, bifunctional antigens primed the mice for a subsequent IgG-PFC response elicited only by a trifunctional antigen (comprised of one haptenic and two carrier epitopes); CFA alone was not sufficient for priming. In guinea pigs, however, bifunctional antigens have induced IgG, as well as IgM, antibody; the difference between murine and cavies was attributed to the different species used in the experiments. It should be pointed out, however, that the species difference was not the only variable; in guinea pigs, bifunctional antigens were injected into the footpads.

The present investigation started by evaluating the contributions of several parameters known to influence

immunogenicity of a compound in mice. We tested four such parameters: size and dose of the antigen, genetic composition of the subjects, and route of immunization. Some other parameters include phylogenetic foreignness (to the subject), charge, structural and chemical complexity, physical form (denatured-native or soluble-aggregated), and optical configuration of the antigen. The specific aim of the experiments was to get a murine IgG-PFC response to the simple synthetic bifunctional antigens. The bifunctional antigens are very small in size (MW of DNP-sac-Tyr-ABA = 688, MW of DNP-Pro_{22}-Tyr-ABA = 2712) and very simple in structural and chemical complexity (only one haptenic and one carrier determinant per molecule,separated either by a flexible chain of 6-aminocaproic acid or by a rigid spacer of 22 proline residues) with respect to other antigens commonly used.

Effects of different routes of immunization were tested first, based on the results of different groups of investigators (see the Introduction). Changing the route of immunization from intraperitoneal to footpad, in Balb/c mice, led to a significant primary IgG-PFC response in the local draining popliteal and inguinal lymph nodes (Fig.1). The response was predominantly of the IgG class (116 IgG-PFC versus 15 IgM-PFC),which is typical of the response to protein antigens.Splenic responses to the trifunctional antigen,however, even after two i.p. injections,were predominantly of the IgM class: 10,000 IgM-PFC/spleen versus 1,605 IgG-PFC/spleen (26). Boosting with the same antigen resulted in a secondary re-

sponse: Higher IgG-PFC and an earlier peak of the response (Exp.7). At the time of the local response in the lymph nodes, there was no significant number of IgG-PFC in the spleen. Some of the antigen injected in the footpads would be expected to travel to the spleen via blood circulation (see the Introduction), but despite the presence of antigen and an ongoing response in a distal place in the same animal, no IgG-PFC were found in the spleen in situ.A few IgG plaques observed in later days were probably due to circulating plasma cells.

Several bifunctional antigens differing in size and spatial distance between the functional groups were also tested in the footpads (Fig.2 and Table I). Results indicated that the distance between the haptenic and carrier determinants was more important than the size in affecting the immunogenicity of the bifunctional antigens, confirming the guinea pig results (30). Differences among the groups were always quantitative, not qualitative; all bifunctional antigens tested elicited IgG-PFC responses. Thus, an optimal distance between the functional groups was required for strong responses as given by DNP-Pro_{22}-Tyr-ABA and DNP-Pro_{16}-sac-Pro_{15}-Tyr-ABA (in this case, because of the flexibility introduced in the middle of the compound by the -sac- moiety, the molecule can bend, bringing DNP and Tyr-ABA functional groups together). Both DNP-sac-Tyr-ABA and DNP-Pro_{31}-Tyr-ABA gave smaller responses. Although DNP-Pro_{22}-Tyr-ABA induced a better response than DNP-sac-Tyr-ABA, a much bigger antigen,DNP-Pro_{31}-Tyr-ABA,

was no better than the first one. In fact, it induced a response similar to that elicited by the DNP-sac-Tyr-ABA.

These findings confirmed the results of guinea pig experiments (30). In the investigation of the relationship of the spatial arrangements of functional groups to the immune response, it was found that haptenic and carrier determinants of an antigen should be at an optimum distance from each other in order to evoke a humoral response. Thus, whereas DNP-Pro_{10}-Tyr-ABA and DNP-Pro_{22}-Tyr-ABA gave good responses, DNP-Pro-Tyr-ABA and DNP-Pro_{31}-Tyr-ABA did not. The model -that optimally placed T and B cell determinants are required for effective T and B cell interaction- that emerged from that study was tested further. It was argued for the antigen DNP-Pro_{31}-Tyr-ABA that, if the distance between the functional groups was the most important factor in not evoking an antibody response, then bending the molecule in the middle would lead to antibody production. Accordingly, another antigen was synthesized with about the same number of proline residues, but with an additional flexible residue (-sac-) in the middle: DNP-Pro_{16}-sac-Pro_{15}-Tyr-ABA. When tested in guinea pigs, this new antigen induced a humoral response and DNP-Pro_{31}-Tyr-ABA again did not (203).

Since the present experiments were carried out with Balb/c mice whereas earlier work was done with A/J mice, the influence of strain differences was assessed by immunizing Balb/c animals i.p. with a series of DNP-spacer-Tyr-ABA compounds (Exp.2 and Fig.2). All compounds gave similar results;

IgM-PFC responses 2-5 fold over the CFA controls and no IgG-PFC. Thus, no differences in splenic PFC were observed between the two strains. During the course of Experiment 2, popliteal, inguinal, mesenteric, peri-aortic and parathymic lymph nodes were also assessed for anti-DNP plaques, and except for a very weak IgG-PFC response (20 IgG-PFC/10^6) in the parathymic nodes, no IgG-PFC were found. IgM-PFC responses were also weak, 5-10 per 10^6 cells. Thus, it was concluded that no significant responses was generated in the lymph nodes following i.p. immunization.

Next, a broad range (0.5-500 μgr/mouse) of different doses of bifunctional antigen was injected to try to circumvent any low or high zone tolerance (Fig.3). All the different doses of antigen gave similar IgM-PFC values and no IgG-PFC. The reason for the lack of anti-DNP IgG-PFC in i.p. primed mice was not dose related. It should be pointed out that in i.p. and f.p. primed animals, spleens and lymph nodes were periodically assayed for anti-ABA PFC; neither direct nor indirect plaques were found. Thus, it was concluded that ABA and DNP groups were acting as T and B cells determinants, respectively, just as indicated by previous work (25,26). The T cell dependent (TD) nature of the bifunctional antigens was further supported by the isotypic analysis of the IgG-PFC response in the lymph nodes (Fig.5). The results were typical of a TD antigen (191): a higher ratio of IgG1, intermediate levels of IgG2a+b, and very little IgG3.

Recruitment of helper T cells induced in the lymph nodes of f.p. primed mice to the spleen was tried by challenging the mice i.p. 10 or 30 days after the f.p. injection (Exp.2). That protocol was not successful in inducing an IgG-PFC response in the spleen. Either the helper cells were not recruited or i.p. priming had induced more suppressor cells than helpers, even in f.p. primed mice. In the reverse situation, f.p. challenge of the animals after they had been i.p. primed failed to give IgG-PFC in the nodes (Table V), implying either the lack of generation of helper cells in the presence of suppressor cells or the prevalence of suppressor cells over the helper cells. Furthermore, the rather short duration of the IgG-PFC response(Fig.1) hints at the generation of suppressor cells in the lymph nodes of the f.p. (alone) primed mice as well. Conversely, the results of a previous work (26) implied that helper cells were in fact induced in i.p. priming, because i.p. injection with bifunctional antigens (in contrast to CFA controls) had been efficient in priming the mice for an IgG-PFC response to the trifunctional antigen. Collectively, these findings suggest that both helper and suppressor cells are induced in response to bifunctional antigens injected either i.p. or f.p.; and the outcome of different routes of immunization depends upon the ratio of helpers to suppressors induced in spleen versus lymph nodes. However, these conclusions are neither new nor restricted to the bifunctional antigens. With conventional antigens, it has been shown that both helper and

suppressor cells are induced in various different lymphoid organs after an antigenic challenge (204). The type of immune response depends upon the ratio of helper and suppressor cells induced (205); and, finally, suppressor cells tend to segregate in the spleen (206,207).

The last parameter of the bifunctional antigen response tested was the influence of the genetic composition of mice (Table II). With respect to IgG-PFC produced in the lymph nodes after f.p. priming, Balb/c ($H\text{-}2^d$) and A/J ($H\text{-}2^a$) were found to be high responders;C57Bl/6 ($H\text{-}2^b$) intermediate responders; SJL ($H\text{-}2^s$) low responders; and CBA/J ($H\text{-}2^k$), SWR ($H\text{-}2^q$), DBA/1 ($H\text{-}2^q$) non-responders to the bifunctional antigen. Responsiveness was found to follow Mendelian genetics: the cross between high ($H\text{-}2^d$) and non ($H\text{-}2^k$) responsive haplotypes produced intermediate responders(Exp.6). However, responsiveness was not found linked to the I region of the H-2 complex since two strains, A/J and CBA/J, sharing the same haplotype in the I region were found to be different responders.

In summary, four parameters: size and dose of the antigen, genetic composition of the mice and route of the immunization were tested for their effects on the immunogenicity of the bifunctional antigens. It was found that route of immunization and genetic composition of mice were the most important factors.The spatial relation of the functional groups to each other was more critical than the size of the antigen. And, finally, the dose of the antigen did

not affect the immunogenicity of the bifunctional antigen injected i.p.. Footpad,but not i.p., immunization enabled the bifunctional antigen to elicit an IgG-PFC response. The possible reasons for this dichotomy can be grouped into three: physiological differences between the two organs, different modes of antigen presentation, and different subpopulations of lymphocytes in spleen versus lymph nodes.

Since the first factor, which includes the spleen's and lymph nodes' basic anatomy, their connections to the lymph and blood circulation, and lymphocyte and antigen circulation within the two organs has been already discussed in the Introduction, it will not be analyzed further. A few points are worth repeating, however. Antigens which reach the spleen first circulate in blood stream where they might interact with pre-existing (if any) immunoregulatory cells or factors, and in their journey to the spleen they are further processed in the liver. Based on the current literature, subcutaneously injected antigens seem to reach the local lymph nodes only via the lymphatic vessels. Follicular dendritic cells(FDC) in lymph nodes seem very effective in capturing the antigen and focusing cellular interactions, thus providing a micro-environment that is probably critical for very small and simple antigens, like those used in this study. FDC retain the antigen on their plasma membrane surfaces for a long time and are probably responsible for local memory. Furthermore, entry of the lymphocytes into the lymph nodes at high endothelial venules (HEV) is very specific. Only metabolically

active and mature lymphocytes adhere to HEV and thus enter the node. Erythrocytes, polymorphonuclear leucocytes and immature bone-marrow and thymus cells do not adhere to HEV. This selective entry populates the nodes with more mature lymphocytes than spleen (208).

The crucial role of macrophages and other antigen presenting cells in the immune response will not be discussed here in detail (the subject is well covered in Ref.209).They are required for the induction of T helper cells (210) and it has been shown both in vivo (106) and in vitro (105) that their depletion from the interacting cell populations favors the induction of T suppressor cells. They are a heterogeneous cell population,and what type of accessory cell presents the antigen to lymphocytes could be a factor in determining the outcome of an immune response (the examples other than the following are discussed in the Introduction Section). In contact sensitivity experiments (211,212), mice intravenously primed with TNP-coupled spleen cells, splenic macrophages or peritoneal-exudate cells failed to give a contact sensitivity response following skin painting with picryl chloride. In contrast, mice primed in the same way with DNP-coupled Langerhans or splenic dendritic cells gave a response which was comparable to those primed with picryl chloride subcutaneously (the latter is the conventional method of priming for contact sensitivity). Furthermore, the positive results of antigen presentation by Langerhans or dendritic cells were not affected by either concomitant injection of TNBS (a strong

inducer of DTH T suppressor cells specific for TNP) or by transfer of contact sensitivity by spleen cells from DNP-Langerhans primed mice into normal mice. The latter protocol is usually expected to result in a reduced response due to feedback suppression induced in the non-immune cell population by the added primed cells (213). Those results indicated that antigen-presenting Langerhans or dendritic cells induced at least one additional type of regulatory cell (other than helper cells) which actively opposed the action of suppressor cells. This cell is referred to as a contrasuppressor (214,215).

An interesting idea suggested by Gershon (216) is that the type (primary or secondary) of the immune response induced by an antigen depends on the system's strategy in dealing with the suppressive and helper signals induced by the antigen itself. If the suppression is simply bypassed, such as by elimination of suppressor cells by anti-Ly2 serum (or by coupling the antigen to potent immunogenic carriers), a primary response is always obtained regardless of boosting regimens. On the other hand, if the antigen induces contrasuppressor cells, which actively combat the suppressor cells, then a secondary response is obtained upon subsequent challenge . In addition, contrasuppressor cell induction in micro environments could be a mechanism of getting a local response in the face of systemic suppression. It is appropriate to reconsider the results of the experiments with bifunctional antigens in the context of the above-mentioned ideas.

It can be postulated that in i.p. immunization these antigens are presented to splenic lymphocytes by splenic or peritoneal macrophages and thus induce suppressor cells, but no contrasuppressor cells. As a result, no secondary responses are obtained in later challenges and the balance tips toward suppression. In contrast, f.p. immunization induces a local response in the lymph nodes, probably as a result of antigen being presented to the resident lymphocytes by a Langerhans or Langerhans-like cell. In that case, they would be resistant to the suppressive signals of level one suppressor cells, such as feedback suppressor cells (Exp.10,control groups). And, they could be responsible for the induction of secondary responses in the nodes(Exp.7), but they would be affected by level two suppressor cells (Exp.9,10,11).

The third possibility to account for the dichotomy of results obtained by i.p. versus f.p. immunization is the different lymphocyte population pattern in these organs. Firstly, the mere ratio of numbers of T and B lymphocytes is different (217). Ratios of lymphocytes in spleen versus lymph nodes for T cell are 35% vs. 77%, and for B cells 38% vs. 18%, respectively. Secondly, as mentioned before, lymph nodes are populated with more mature lymphocytes than the spleen. A recently defined alloantigen Lna-1 (lymph-node alloantigen-1) is expressed on both T and B cells in lymph nodes but not on spleen cells in unprimed mice (218). However, normal spleen cells can be induced to express the Lna-1 marker by antigenic stimulation. Another consideration

is the preferential homing of suppressor cells to the spleen, as previously mentioned.

The reports (183-185) indicating the dominance of Lyb 3(+) B cells in the lymph nodes but not in the spleen prompted the testing of bifunctional antigen in the footpads of CBA/N (♀) x Balb/c (♂) F_1 hybrids (Fig.4). Lyb 3,5(+) B cells represent a more mature subset of B lymphocytes, whereas Lyb 3,5(-) cells resemble immature B cells found in neonatal mice (186). The statistically meaningful ($p<0.001$) difference between the results obtained in males (exhibit the CBA/N xid defect) and females (phenotypically normal) tempts one to postulate that differences in Lyb 3(+) B cell numbers in spleen versus lymph nodes may at least partly account for the observed responses. However,some caution is warranted here, because while unresponsiveness of CBA/N mice is most readily apparent with TI-1 antigens, their responses to TD and TI-2 antigens are subnormal as well. Thus, they give diminished responses to particulate antigens (219,220) and hapten-carrier conjugates in vivo (221), and also produce low affinity antibody (222). They fail to respond to some haptens, such as phosphoryl choline, coupled to immunogenic carriers in vivo (223), and fail to give an in vitro primary response to several TD antigens (224). The differences between the CBA/N responses to PC- and TNP-conjugates (KLH, OVA or mycobacteria, all TD carriers) in terms of PFC production (223) or the transplantation resistance in recipient CBA/N mice towards PC-specific transferred

B cells (225),tie in with the notion of a maturational arrest in CBA/N mice. Thus, while most TNP-specific clones can be found very early in ontogeny (216), PC-specific clones (227, 228),and clonal selection and expansion to dominance of the TEPC-15 idiotype associated with the PC response, occurs at 7 to 10 days of life (229). Nevertheless; the lack of response to PC in CBA/N mice is not absolute, in boosting regimens with PC-KLH (230) or in some primary challenges, i.e., to PC-LPS (231), they have been shown to respond. However, the response is marked by a total absence of IgG3 production. A careful investigation of the CBA/N response to hapten-carrier conjugates (187) has also revealed a similar isotypic response pattern. Impaired responses to TD antigens in defective mice were due to diminished IgM and IgG3 responses; IgG1 and IgG2 responses between the defective and normal mice were similar. The conclusion derived from Experiment 6-A (obtained from results of xid defective male and normal female hybrids), that local IgG responses to bifunctional antigens in the lymph nodes are partly due to the surface phenotype of B cells (95-100% of B cells are of Lyb 3+ type in normal mice) residing in the nodes, is supported by the above considerations in spite of inherent impaired responses of xid defective mice toward TD antigens. First, the B cell determinant of bifunctional antigen is DNP (highly cross reactive with TNP), a hapten towards which the number of clones of B cells is not limited and for which clonal selection occurs very early in ontogeny. Second, the lymph node response to bifunc-

tional antigens consists mainly of IgG1 and IgG2a+b classes (Fig.5),which were shown to be unaffected in xid mice.

Since there were some differences in the lymph node responses between male (phenotypically and genotypically normal) and female (phenotypically normal, but has one X chromosome with a recessive xid gene from a defective father) progeny from the control cross (Balb/c(♀) x CBA/N(♂)), experiment 6-B was carried out to distinguish between the two explanations: sex difference or gene dosage effect. It was assumed that if the differences were due to a gene dosage effect, then there would be a greater difference between the responses of female and male hybrids to DNP-Ficoll, a TI-2 antigen,than to TNP-LPS (TI-1 antigen) and DNP-OVA (TD antigen). Male and female hybrids responded to DNP-Ficoll equally well, and there was no significant difference between their responses to DNP-OVA and TNP-LPS either (Table III). Since control female mice from the cross Balb/c (♀) x CBA/J (♂)were not tested to compare with Balb/c (♀) x CBA/N (♂) female F_1 mice, it is difficult to rule out the influence of gene dosage .CBA/J mice are genotypically identical to CBA/N mice, but do not have the xid gene.

The experiment was not decisive to rule out either one of the two explanations: sex difference or gene dosage effect. The experiment, nevertheless, gave an interesting result in that TNP-LPS did not induce a PFC response in the lymph nodes (spleens gave PFC in the same mice). Several reports indicate that LPS activation of B cells does not require the

presence of Lyb 3,5(+) B cells (232-234), and B cells responding to LPS are different from B cells responding to TD or TI-2 antigens in size (235) and in drug-azathioprine-sensitivity (236,237). However, there is no report which indicates that hapten coupled LPS cannot activate hapten specific Lyb 3,5(+) B cells. Thus, the results (Table III) that TNP-LPS did not evoke a response in the nodes where nearly all of the B cells are of Lyb 3,5(+) phenotype and was efficient in eliciting an IgM-PFC response in spleen where half of the B cells are of Lyb 3,5(-) phenotype, suggest that in hapten specific responses LPS activates only Lyb 3,5(-) cells. It should be stressed that in Experiment 6-B, a small dose of TNP-LPS was used to avoid non-specific mitogenic activation of B cells, so the possibility exists that Lyb 3,5(+) cells can be activated by LPS at mitogenic doses.

In summary, three factors have been discussed in their possible influences on the local immune responses in the lymph nodes and on the systemic immunity reflected in the spleen. In view of the agreement of the results of this investigation with the above consideration of these factors, it is felt that each of the three factors (different subpopulation of lymphocytes, mode of antigen presentation and physiological differences between the spleen and the local lymph nodes) contributes to the different immune responses observed at these organs to the bifunctional antigens.

After establishing a local IgG-PFC response in the lymph nodes by footpad immunization and testing the contribu-

tions of several factors to the status of immune responses in spleen and in nodes, investigation focused on the consequences of initial i.p. priming on the subsequent f.p. challenge with bifunctional antigens. In the course of the experiments, it was found that i.p. immunization with DNP-sac-Tyr-ABA induced antigen specific suppressor cells which would,in turn, suppress the IgG-PFC response in the lymph nodes induced by f.p. challenge with DNP-Pro_{22}-Tyr-ABA in the same animal or in recipient mice. The epitope responsible for the suppression induction was Tyr-ABA and the suppressor cells behaved in a carrier-induced hapten-directed fashion. Inhibitory activity of Tyr-ABA induced suppressor cells on the DNP-OVA response raised the possibility of an idiotypic connectance between anti-ABA and anti-DNP responses in Balb/c mice.

Several protocols (changing the dose of antigen, priming the mice in the footpads before i.p. challenge to help the recruitment of more helper cells) were tried to get an IgG-PFC response to bifunctional antigens in the spleen, without success. As a last resort (Exp.8) mice were injected with low or medium doses of cyclophosphamide (CY) before i.p. immunization. It has been suggested that precursors of level one suppressor cells, but not of level two suppressors,are CY sensitive (118). Inability of CY treatment to change the splenic immune response (Table IV) suggested that if bifunctional antigens were inducing suppressor cells in the spleen, they were not level one suppressor cells.

Induction of effector suppressor cells by a single

i.p. immunization with bifunctional antigen was shown in Experiment 9. Such effector cells were generated as early as one week after initial priming and persisted for two to three weeks,but were absent by the fourteenth week. They suppressed the IgG-PFC response in the lymph nodes following f.p. challenge in the same animal.Since the IgM-PFC response to bifunctional antigens in the node was very small, no statistically-meaningful conclusions could be made. Suppressor cells were antigen specific inasmuch as the response to BSA was not affected.

Spleen cells from i.p. primed mice were able to transfer the suppression into f.p. primed recipient mice (Fig.6). The observed suppression cannot be explained by induction of level one effector feedback suppressor cells in the recipient mice in response to transferred primed lymphocytes. First, the recipients were already immunized one or two days before. Second, the immunization was done in the footpads, probably inducing contrasuppressive cells in the local lymph nodes (discussed earlier). It has been shown that contrasuppressor cells can combat the suppressor cells in vitro (238) and in vivo, permitting the expression of humoral (239) and contact sensitivity (215) responses. It has been postulated that members of the feedback suppression and contrasuppression circuits interact during an immune response (240), and a projection was made that only level two suppressor cells may override the effectors or effects of contrasuppression (214). Third, in a similar feedback sup-

pression system (241), it has been shown that inducer cells in the transferred cell population are the primed B cells and induction of feedback suppression effectors in the recipient mice is dependent on the immunoglobulin structures present on activated B cells (242,243). In the present investigation, neither a pure population of B cells (Exp.12) nor T cell-depleted suppressed spleen cells (Exp.11) induced any suppression in the recipient mice. However, T cells from such spleen cells actively transferred suppression. It can be argued that in the panning experiment 12, the recovery of B cells at room temperature from anti-immunoglobulin coated plates might have resulted in the capping of surface immunoglobulins: however, elimination of T cells with anti-θ plus complement treatment would not remove such structures from the surface of B cells.

The affects of suppression induced by i.p. priming with bifunctional antigens are evidenced by the diminished IgG-PFC response. There is no significant difference between the primary and secondary IgM-PFC responses either in spleen (26) or lymph nodes. Similar results have been observed in hapten-carrier systems by Tada(109) and Herzenberg(244). Differential regulation of the immune responses by specific T cells, however, has been observed by many investigators. Examples include helper T cells specific for allotype (245), isotype (246-248) or idiotype (135,249,250); and suppressor T cells specific for allotype (251), isotype (252,254) or idiotype (255,256).

Since the object of the present investigation was not to investigate the surface phenotype of the suppressor cells induced by bifunctional antigens, only the T cell nature and antigen specificity of these suppressor cells were verified (Experiment 11). After demonstrating the antigen specificity of the suppressor cells (Table IV, Fig.7), their binding capacity to the carrier epitope was tested in Experiment 12. Previously, it had been shown that ABA suppressor cells can bind to ABA-Protein coated plates and be recovered without loss of their functional activity (128). Results (Table V) showed that cell populations that bound to ABA-plates were highly enriched for suppressor activity (95-100%), but not all suppressor cells bound to the antigen. This result could be interpreted in two different ways: either binding to the plate was inefficient so that not all of the antigen specific suppressor cells were bound, or there was a second population of suppressor cells which were not antigen specific. Since panning of non-ABA-plate-adhering suppressor cells on idiotype -coated plates was not tested, it can only be hypothesized on the basis of our current understanding of immunology that this second set of suppressor cells was idiotype specific.

The haptenic (DNP) and carrier (Tyr-ABA) determinants of the bifunctional antigens were tested (Exp.13) for their ability to induce suppression. When injected separately as DNP-Lysine or Tyrosine-ABA into the mice, only Tyr-ABA induced suppressor T cells (Fig.8). Induction of suppressor cells with Tyr-ABA had been reported earlier in mice (128)

and in rats (257).

In the last experiment, the target of suppression was evaluated: would suppressor cells induced by mono- or bifunctional antigens suppress anti-ABA responses evoked by ABA-BSA or anti-DNP responses evoked by DNP-OVA ? Since they were induced by the ABA group, the expected result was that they would suppress only anti-ABA responses. However, 10-40% suppression of the anti-ABA response was not very significant when compared to 95-100% suppression of the anti-DNP response evoked by DNP-Pro_{22}-Tyr-ABA. In a previous study (128), spleen cells from Tyr-ABA i.p. primed A/J mice had also suppressed at similar levels: 40-50% of the anti-TNP response to TNP, ABA-KLH. In that study, the ABA group was presented as a haptenic determinants also. The moderate levels of suppression in both experiments raised the question of the necessity of providing ABA groups as the sole carrier epitope in order to get high levels of suppression. The possibility existed that extra help was being generated in response to the carrier proteins, BSA or KLH, used in these experiments. The best solution to these considerations was to use an antigen whose haptenic and carrier determinants were both ABA. Such a symmetrical bifunctional antigen, ABA-Tyr-Pro_{10}-Tyr-ABA, was shown to induce anti-ABA antibodies in the guinea pigs (25). However, this compound when tested in mice, did not elicit any response in the lymph nodes or in the spleen (unpublished data). These results were not surprising in view of the difficulty of obtaining primary anti-ABA responses to ABA

coupled to conventional proteins. A boosting experiment was not performed, and the issue of suppression of anti-ABA responses remained unsolved.

On the other hand, the meager suppression by ABA-Tyr of the anti-ABA responses to ABA-protein draws attention to the "epitope-specific suppression" described by Herzenberg (244) in "carrier/hapten-carrier" immunized mice. In that system, carrier-induced and carrier-specific suppressor cells abrogated the IgG anti-hapten responses to a hapten presented on that carrier, but did not affect the IgM or IgG responses to the carrier itself. Although no satisfactory mechanism was provided as to how this regulatory system really works, enough additional examples have been reported by Rajewsky (258), Mitchison(259), Tada(113) and Rausch(260) to validate its reality. Features of the suppression induced by monofunctional "[Tyr-ABA, i.p.]/DNP-[-Pro_{22}-Tyr-ABA, f.p.]" and bifunctional antigens agree with the features of suppression described in that model. Consequently, suppression of anti-ABA responses should not be expected in the context of the carrier "epitope-specific suppression" model, and the insignificant ($p>0.4$) suppression observed in the present study is consistent with this. There is a small problem, however, with the suppression induced by i.p. priming with bifunctional antigen. That is, the carrier epitope is injected coupled to hapten, but not alone. It can be argued, however, that because there is no induction of a hapten specific IgG response in the spleen by bifunctional antigen, it acts just like the

monofunctional antigen in "epitope-specific suppression". Then the question arises: would they act differently in a situation where there is an IgG response after the first immunization with bifunctional antigen. The solution is, of course, to perform both injections in the footpads. When this was tested, two sequential f.p. injections of the bifunctional antigen gave a perfect secondary response: 250 IgG-PFC (Exp.7); the "monofunctional, f.p./bifunctional, f.p." protocol again resulted in suppression: 40 IgG-PFC, 84% suppression (unpublished data). So the answer to the question is affirmative; when there is an initial IgG anti-hapten response, bifunctional antigen acts differently and primes for a secondary response rather than inducing suppression. But in the absence of an initial IgG response, it induces an "epitope-specific suppression". Another reason for the induction of a secondary response after two footpad injections of bifunctional antigen might be the interference of contrasuppressor cells induced after the first injection (discussed earlier). However, they should have been induced after the monofunctional (f.p.) injection as well, but in that case suppression was observed.

Experiment 14 gave some interesting results. It should be expected that carrier-induced T suppressor cells of "epitope-specific suppression" must recognize the carrier they are specific for on the challenging antigen in order to suppress. Clearly, the challenging antigen, DNP-OVA, in that experiment does not have any functional ABA epitope, yet the

anti-DNP response is significantly suppressed (p=0.005) (40-50% suppression in both IgM and IgG compartments). This result cannot be explained by the above mentioned epitope-specific suppression. Although the issue of the recognition of new determinants created by the chemical coupling of DNP groups to the rest of the molecule could be raised, it would probably be invalid because in the bifunctional antigen DNP is coupled to the N-proline residue and in OVA almost exclusively to lysine residues (176). Furthermore, perhaps the most exciting result of this investigation vitiates that argument and elucidates the issue: T suppressor cells induced with the ABA group (without encountering the DNP group) were able to suppress (40-50%) both the IgM and the IgG responses to the DNP presented on a carrier (OVA) which did not contain any ABA groups. These results when combined with the observed cross-reactivity of MOPC-460 and 36-60 proteins and the inability to completely remove suppressor cells by antigen panning suggested that i.p. priming with mono or bifunctional antigens might induce both epitope-specific and idiotype specific T suppressor cells in the spleen. In the ELISA assay, rabbit anti-M-460 anti-idiotypic reagent bound to both M-460 and 36-60 protein coated plates. Binding of that reagent to M-460 plates was inhibitable by either M-460 or 36-60, but not by TEPC-15 proteins. Thus, if the idiotype specific T suppressor cells recognize an idiotype shared by anti-DNP and anti-ABA antibodies, selective suppression of that idiotype compartment of anti-DNP responses might be observed in the

absence of ABA groups on the antigen. Evidence for such idiotypic regulation was observed in the results of the plaque inhibition assay by anti-CRI^C antiserum (Table VII-B): 29% of the anti-DNP plaques in the control group were $CRI^C(+)$ and all of the anti-DNP plaques in the suppressed group were $CRI^C(-)$. Thus, only the $CRI^C(+)$ portion of the response to DNP-OVA was suppressed. The shared idiotype of the MOPC-460 and 36-60 proteins is tentatively being called CRI^{c-460}. The relationship is only functionally defined in the present work. Molecular characterization of idiotypic connectance between MOPC-460 and CRI^C idiotypes is being currently investigated with monoclonal MOPC-460 and 36-60 proteins. Although 36-60 is an ABA-binding protein derived from A/J mice (145,146), the minor idiotypic family it represents in the A/J mice has been shown (147,148) to be similar to the major idiotypic family (CRI^C) of anti-ABA antibodies produced in Balb/c mice. The MOPC-460 idiotype is associated with DNP binding antibodies of Balb/c mice.

The possibility of a relationship between DNP and ABA specific responses was hinted earlier by the sequence homology between 36-60 and MOPC-460 proteins (261). Sequence analysis showed a difference of only 4 residues in the first 35 amino acids, including the first complementarity-determining region. Incidentally, the difference between the closely related DNP binding myelomas MOPC-460 and MOPC-315 is 7 residues in the same region. A recent finding indicates such a relationship in A/J mice also (P.V.Hornbeck, personal commu-

nication): anti-TNP antibodies induced either by TNP-KLH or ABA, TNP-KLH bear CRI^{AD8} determinants. CRI^{AD8} is another cross-reactive idiotype expressed on anti-ABA antibodies in A/J mice (164).

Idiotypes shared by antibodies of different specificities can be a unifying theme in the regulation of immune responses. In the face of innumerable environmental stimuli, it gives the system the chance to economize in the numbers of regulatory cells involved. Not surprisingly, the concept of idiotype sharing by antibodies of unrelated specificity is not new, as the first evidence was reported in 1971 (158). In some cases (159,163), the specificities of the antibodies involved are known and in others not (156,164). In the present investigation, the specificities of the antibodies involved (DNP and ABA) are perhaps the simplest of all. The presence of an idiotypic connection between these simple groups might be considered strong evidence for the universality of such regulation.

The last part of the discussion concerns the mechanism of generation of suppressor cells in the spleen in situ after i.p. priming. A consensus of the preceding discussions is that level two (Ts_2) suppressor cells were also induced in the spleen, in addition to level one (Ts_1) suppressor cells. The arguments for this are briefly as follows: their induction was not CY sensitive; full suppression was reached by the first week; they were not completely depleted by panning on antigen plates; they were able to transfer suppression into

primed animals; and their generation did not require a second antigenic stimulation. These findings suggest that there must be some functionally active precursors of idiotype-recognizing level two suppressor cells in unprimed Balb/c mice. In fact, such suppressor cells were reported by Bona(262): when splenic T cells from normal Balb/c mice were added to in vitro TNP-Nocardia water-soluble mitogen stimulated cultures, the number of MOPC-460 (M-460) idiotype positive plaques was reduced. The panning of normal spleen cells on idiotype coated plates removed the suppressor cells, antigenic stimulation of normal mice with M-460 protein amplified the suppressive activity (263), and these idiotype specific Ly2,3+ T suppressor cells acted directly on B cells (264). The reported M-460 Id-bearing but not DNP-binding factors (156) in the serum normal Balb/c mice might be responsible for the activation of these idiotype specific suppressor cells. As to the reason why such idiotype bearing factors (antibodies or T cell products ?) do exist in naive mice, it was postulated that (156) such factors were indeed a reflection of an ongoing immune response to an environmental stimulus, and that some of the antibodies produced in response to a pathogen or normal intestinal flora might bear M-460 idiotype.

Another alternative would be that some endogeneous factor was inducing antibodies that cross-reacted with DNP (or TNP) and expressed the M-460 Id. Originally, this factor was thought to be nucleic acid, because TNP-specific mouse

myelomas cross-reacted with purines and with 5-acetyl uracil (265). Accordingly, the high incidence of these TNP binding myelomas induced by oil was considered to be the consequence of an autoimmune reaction to DNA (265). A careful investigation of these myelomas, however, showed that besides the TNP group, the binding was strongest with naphtoquinones (266). Analogs of this compound were abundant in nature, like vitamin K in the intestinal tract. Since most of the TNP specific myelomas arose in the intestinal tract and were of IgA class, it was thought that naphtoquinones in normal mice were immunogenic and that the antibodies induced against them exhibited the M-460 Id.

As the Discussion Section ends, it would be appropriate to reconsider the basic features of the immunity in mice against bifunctional antigens. The route of immunization is found to be very important: intraperitoneal and footpad injections induce different ratios of immunoregulatory cells and hence different immune responses. The bifunctional antigens injected in the footpads evoke mostly a local IgG-PFC response in the draining popliteal and inguinal lymph nodes. On the other hand, intraperitoneal injection leads to a dominant suppression. Both antigen-specific and idiotype-specific T suppressor cells may be induced in the spleen. Preliminary evidence suggests that idiotype specific T suppressor cells induced by the ABA carrier epitope of the bifunctional antigen also recognize an idiotype expressed on anti-DNP antibodies. Since the ABA and DNP groups are widely used by many

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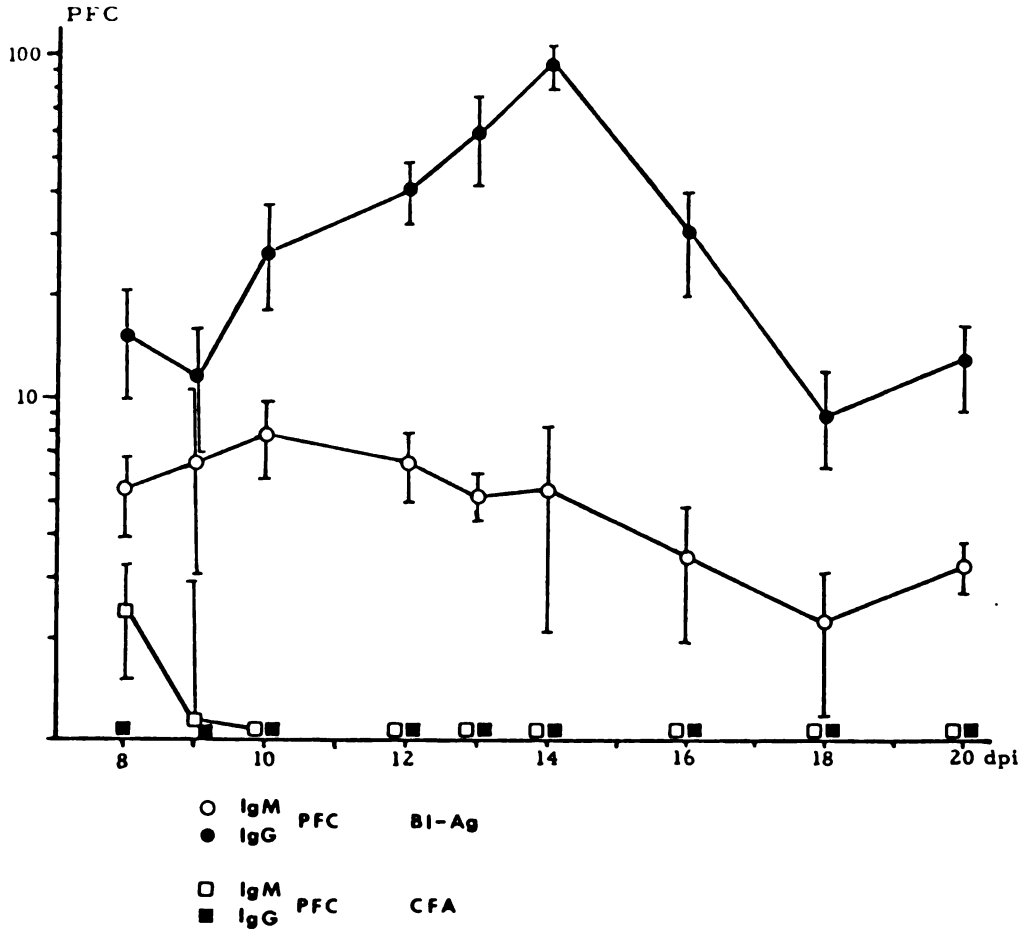

Figure 1. Popliteal and inguinal lymph node response to bifunctional antigens. 100 μgr of DNP-Pro_{22}-Tyr-ABA was injected subcutaneously at the base of the tail and into the hind footpads of Balb/c mice. Control groups received CFA alone. Popliteal and inguinal lymph nodes were assayed for anti-DNP PFC on various days post immunization (dpi). In the figure, PFC/10^6 cells values are represented.

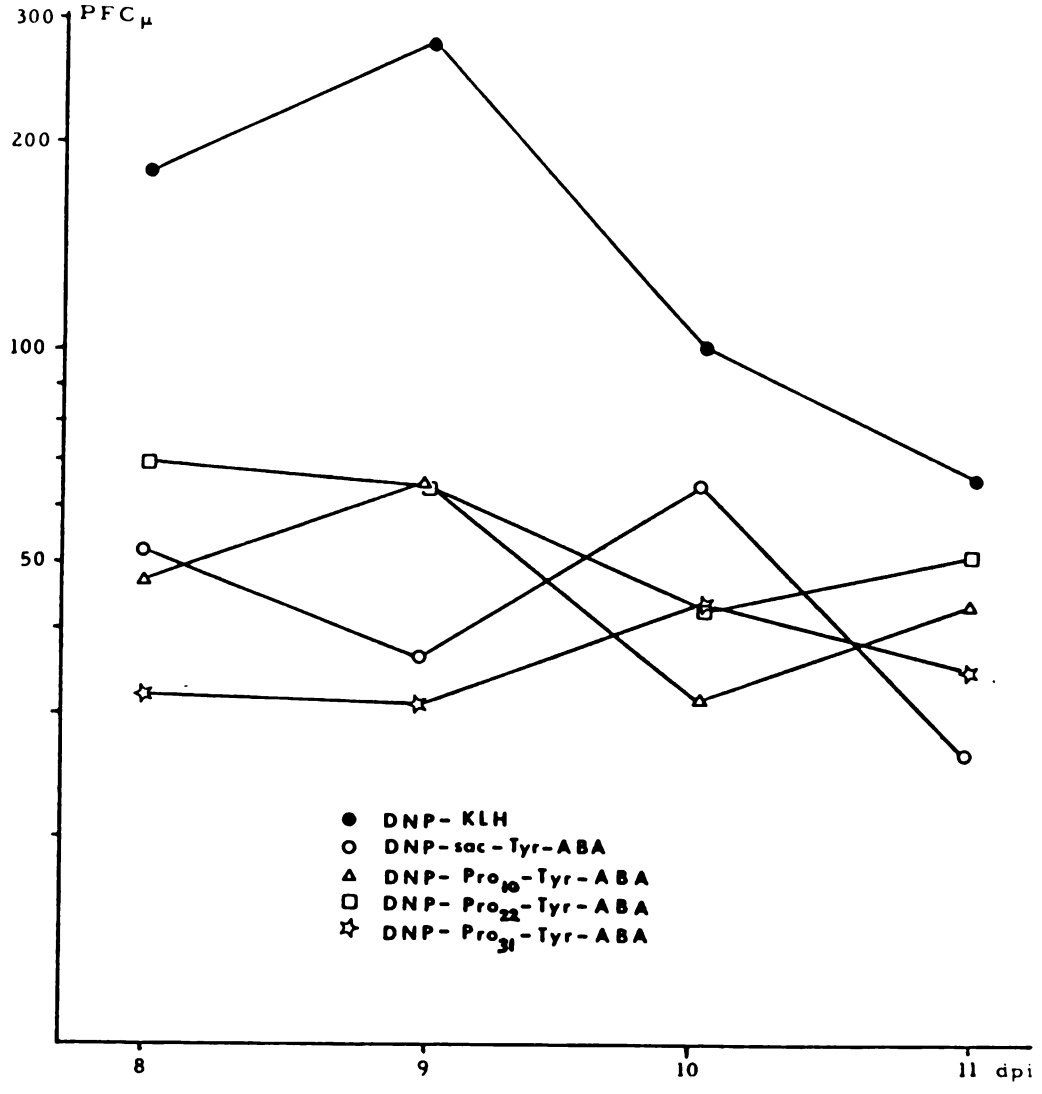

Figure 2. Splenic responses to various bifunctional antigens. 100 μgr of indicated bifunctional antigens and 100 μgr of DNP-KLH were injected i.p., and the spleens were assayed for anti-DNP PFC. IgM-PFC/10^6 values represented in the figure. Spleens from CFA injected control mice gave 20-40 IgM-PFC/10^6.

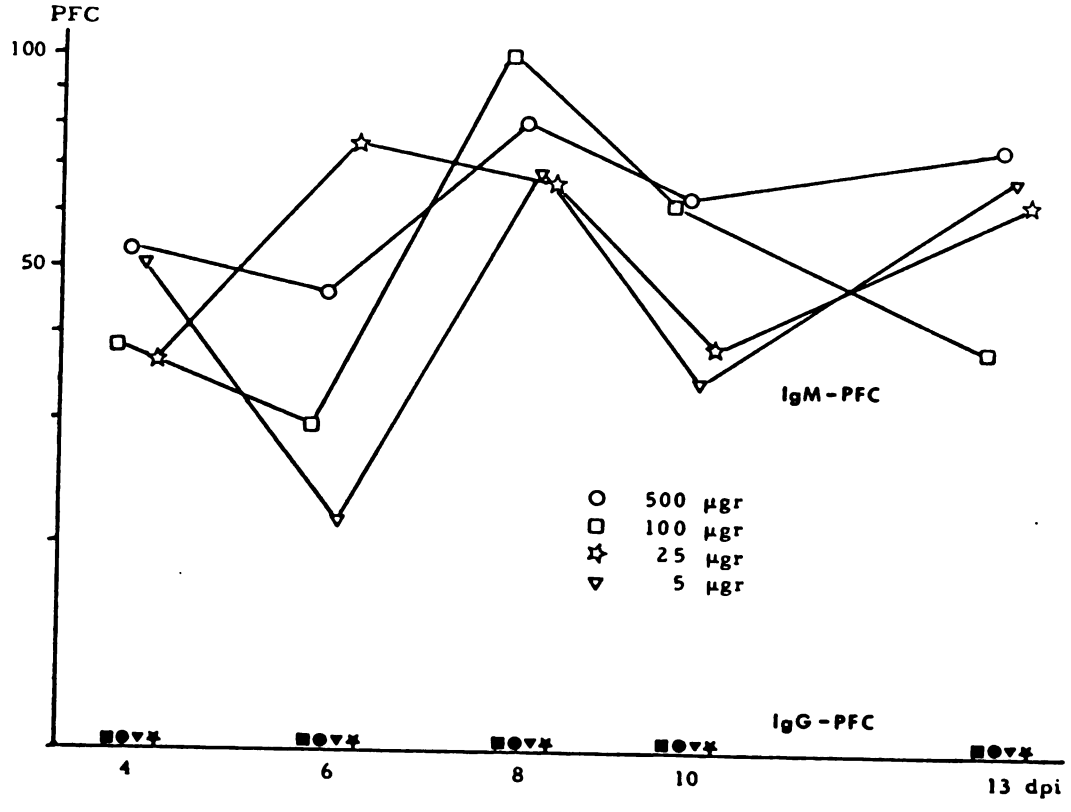

Figure 3. Splenic dose response to bifunctional antigens. The indicated doses of DNP-sac-Tyr-ABA was injected intraperitoneally and the spleens were assayed for anti-DNP PFC. dpi denotes days post immunization and Pfc values are per 10^6 cells.

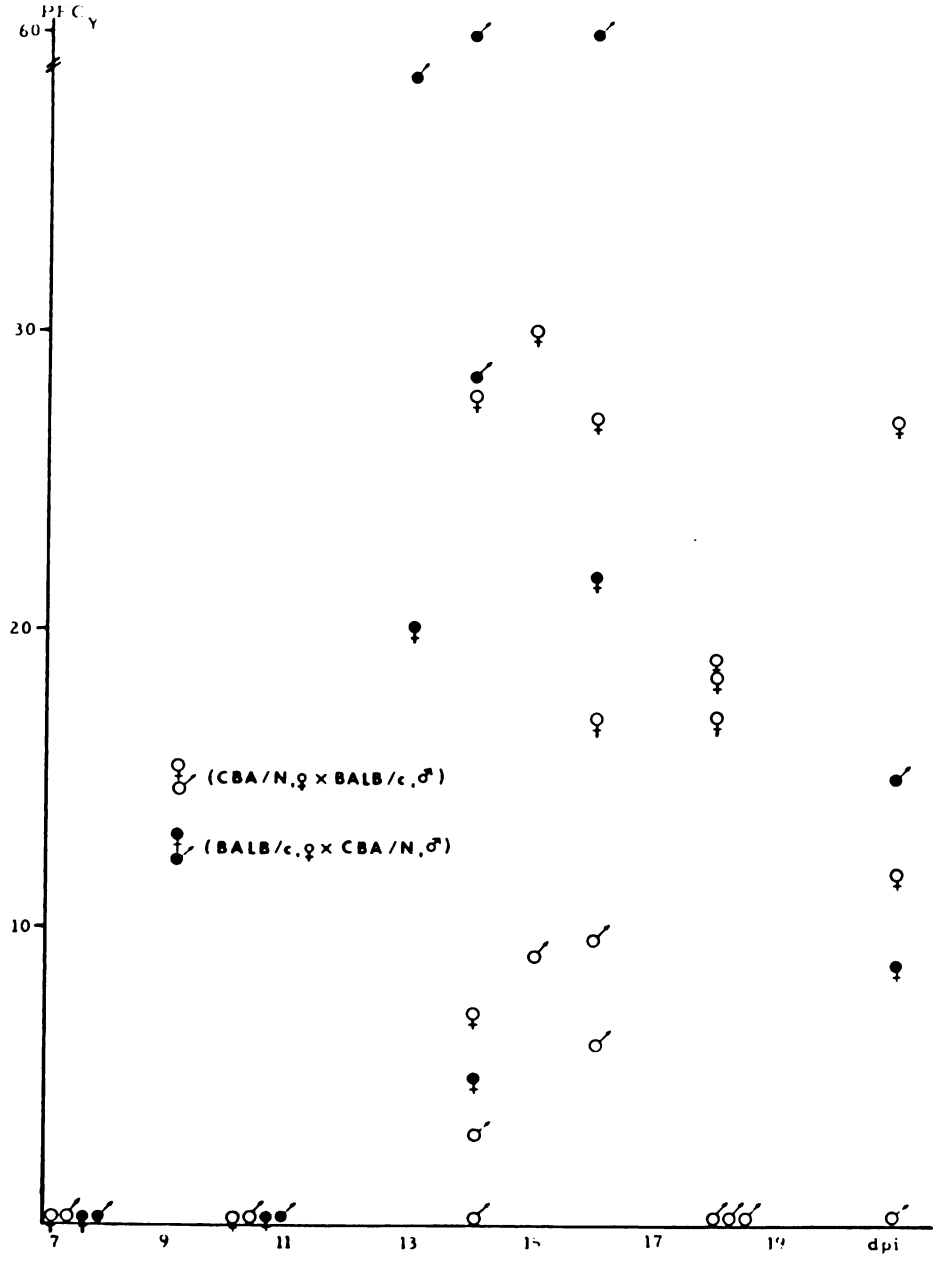

Figure 4. (CBA/N x Balb/c) F_1 lymph node responses to bifunctional antigens. 100 µgr of DNP-Pro_{22}-Tyr-ABA was injected in the footpads, and the draining popliteal and inguinal lymph nodes were assayed for anti-DNP PFC. Data shows individual IgG-PFC/10^6 values for each mice tested.

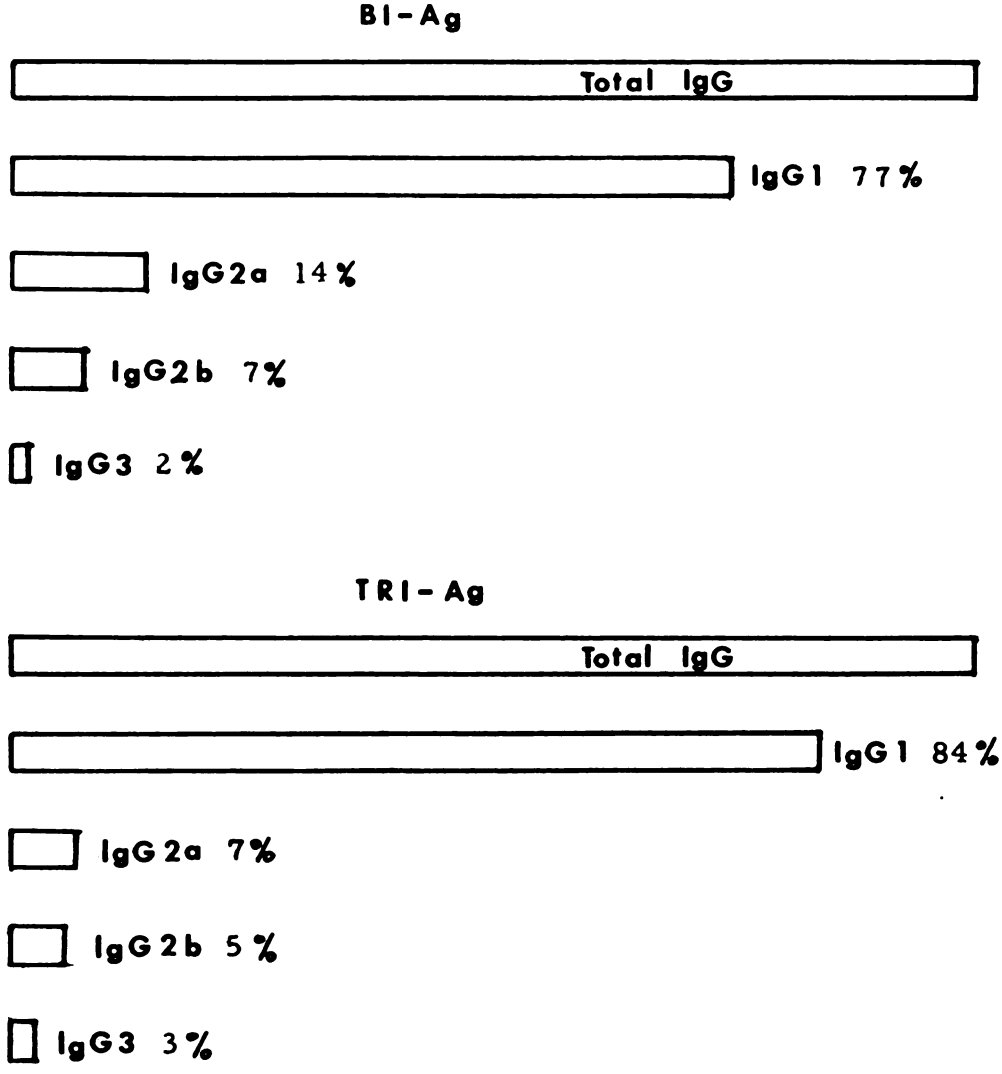

Figure 5. IgG subclass profile of lymph node responses to Bi- (DNP-Pro_{22}-Tyr-ABA) and Tri- (DNP-sac-Tyr-ABA-Pro_9-Tyr-ABA) functional antigens. 100 µgr of each antigen was injected into the hind footpads and 13-14 days later, inguinal and popliteal lymph nodes were assayed for indirect anti-DNP plaques using isotype-specific antisera. Total IgG-PFC/10^6 values for each antigen was normalized to the value of 100.

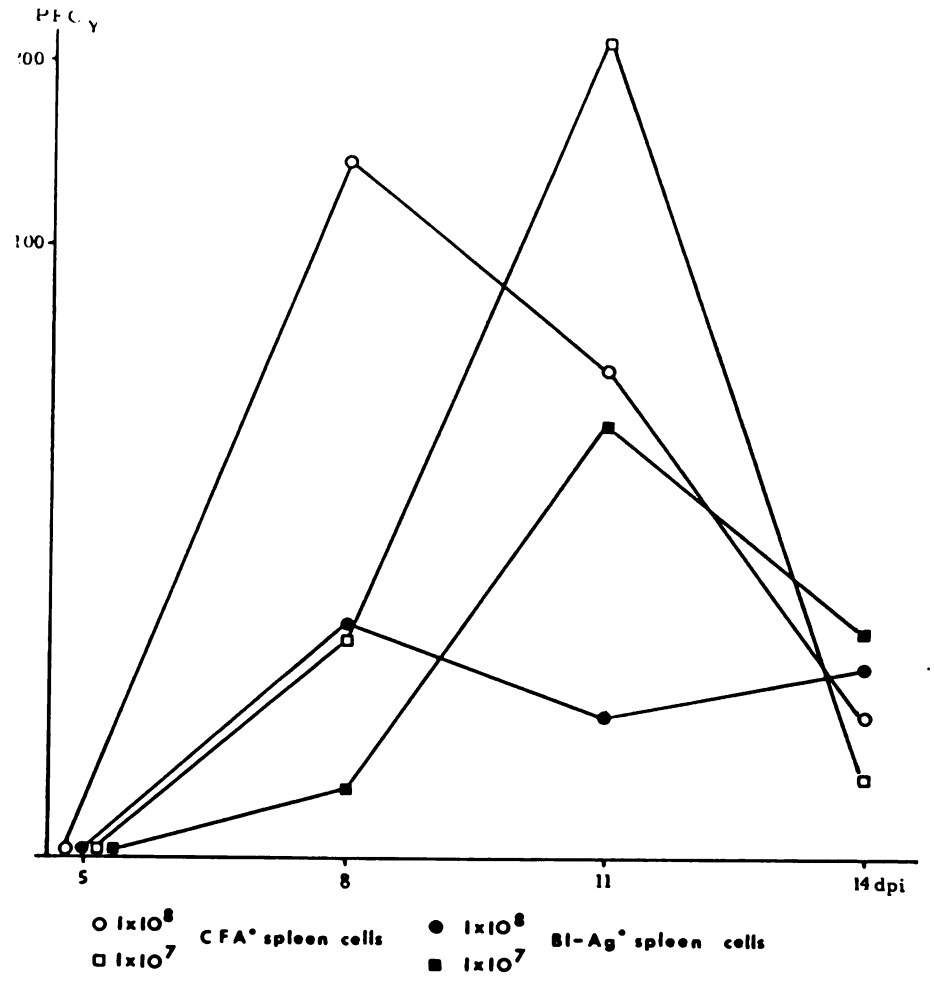

Figure 6. Transfer of suppression by DNP-sac-Tyr-ABA (i.p.) primed spleen cells. $1x10^7$ and $1x10^8$ spleen cells from i.p. primed donor mice were transferred intravenously into recipient mice. The recipient mice were injected with DNP-Pro_{22}-Tyr-ABA, one day before the cell transfer. Popliteal and inguinal lymph nodes were assayed for anti-DNP PFC. The results are expressed as IgG-PFC/10^6.

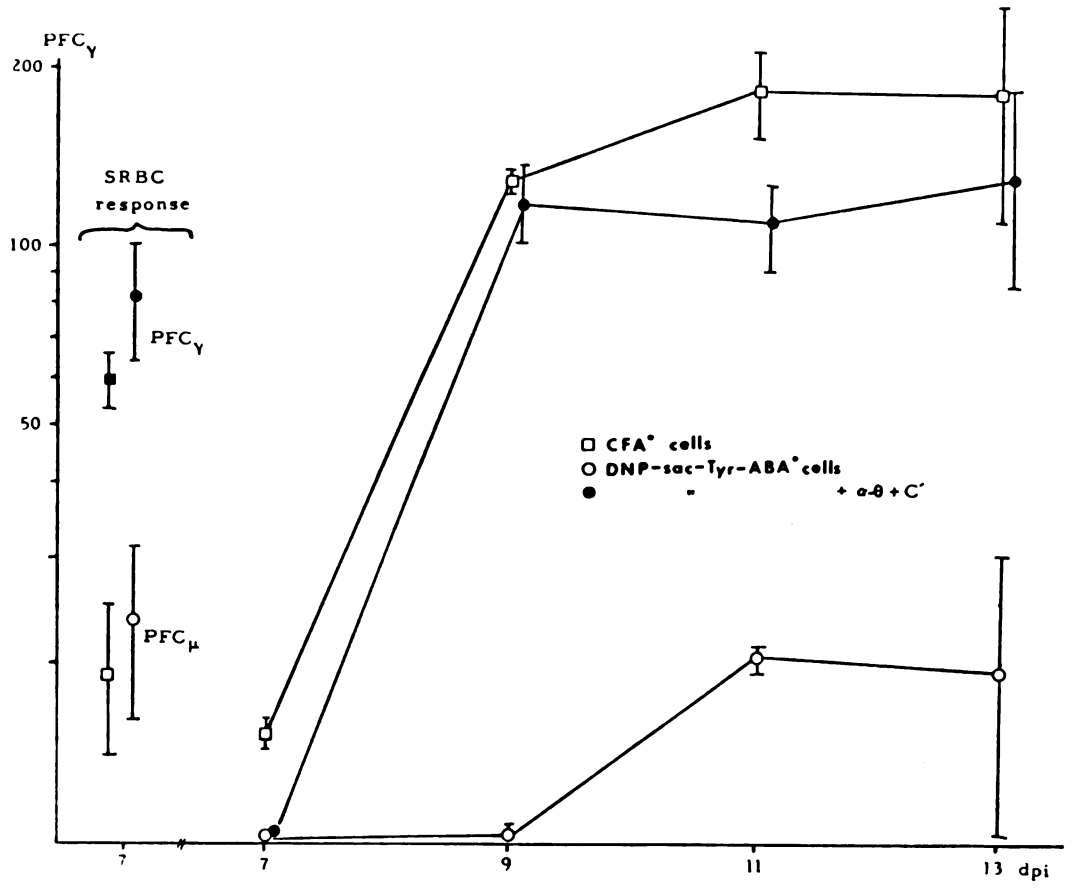

Figure 7. Antigen specificity and T-cell nature of the suppressor cells. Donor mice were i.p. injected with 100 µgr of DNP-sac-Tyr-ABA in CFA (control mice received CFA alone). 2 weeks later, their spleens were removed and T cells were depleted by anti-θ+C' treatment, before they were transferred intravenously into recipient mice. The recipient mice were primed with 100 µgr of DNP-Pro_{22}-Tyr-ABA in the footpads, one day before. 7, 9, 11 and 13 days later lymph nodes were assayed for anti-DNP PFC. T suppressor cells were also transferred into SRBC primed mice along with CFA control cells to test the antigenic specificity of the suppression.

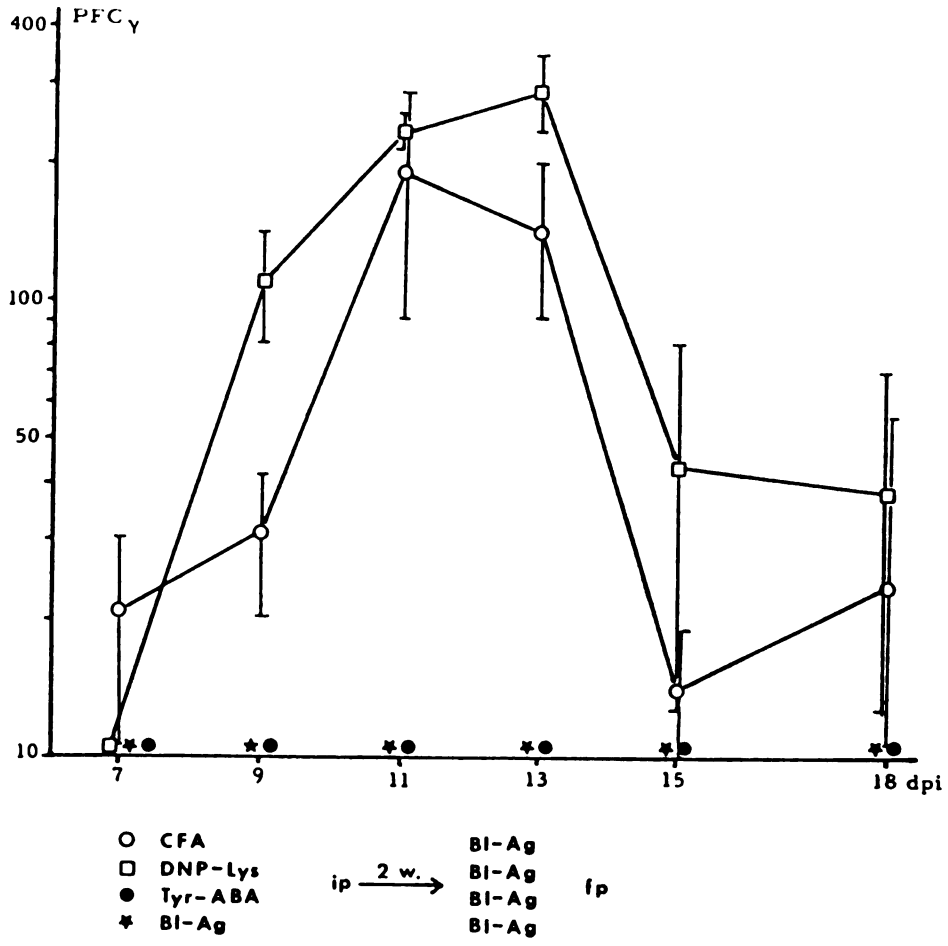

Figure 8. Epitope of the bifunctional antigen responsible for the induction of the suppression. 100 μgr of the indicated compounds were injected i.p. and 2 weeks later DNP-Pro_{22}-Tyr-ABA was injected into the footpads of same mice. 7 to 18 days later lymph nodes were assayed for anti-DNP PFC. IgG-PFC /10^6 values are expressed in the figure.

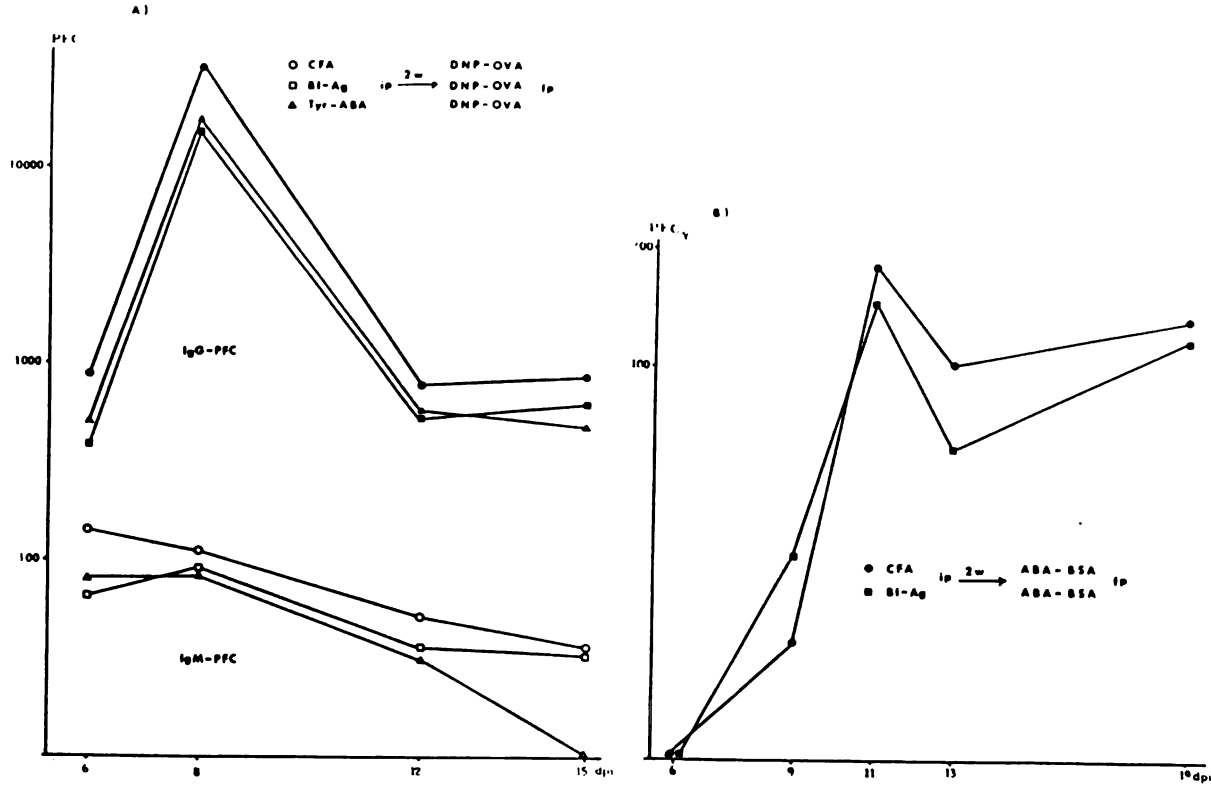

Figure 9. Effect of bifunctional antigen induced suppression on anti-DNP and anti-ABA responses. In figure (A), mice were injected with 100 µgr of either Tyr-ABA or BI-Ag (DNP-sac-Tyr-ABA) intraperitoneally. 2 weeks later, they were challenged with DNP-OVA in the footpads, and the lymph nodes were assayed for anti-DNP PFC/10^6. In figure (B), mice were injected with 100 µgr of BI-Ag i.p., and 2 weeks later they were challenged with 100 µgr of ABA-BSA in the footpads. Anti-ABA IgG-PFC/10^6 values are represented in the figure.

Table I

LYMPH NODE RESPONSES TO DNP-sac-Tyr-ABA, DNP-Pro_{22}-Tyr-ABA AND DNP-Pro_{16}-sac-Pro_{15}-Tyr-ABA

dpi[a]	Antigens[b]	IgM-PFC/10^6 (c)	IgG-PFC/10^6 (c)
12	CFA	8±1 (d)	0
	D-s-R	13±1	2±2
	D-P_{31}-R	19±9	47±8
	D-P_{16}-s-P_{15}-R	27±5	231±23
14	CFA	3±0.2	0
	D-s-R	18±4	11±4
	D-P_{31}-R	18±6	86±35
	D-P_{16}-s-P_{15}-R	18±3	102±20
16	CFA	11±4	0
	D-s-R	20±13	23±10
	D-P_{31}-R	29±2	10±0.1
	D-P_{16}-s-P_{15}-R	28±4	21±2

a) dpi=days post immunization

b) CFA=control mice injected with Complete Freunds Adjuvant alone. D-s-R=DNP-sac-Tyr-ABA, D-P_{31}-R=DNP-Pro_{31}-Tyr-ABA, D-P_{16}-s-P_{15}-R=DNP-Pro_{16}-s-Pro_{15}-Tyr-ABA.100 µgr of each antigen in CFA is injected subcutaneously at the base of the tail and into the footpads.

c) Popliteal and inguinal lymph nodes are assayed for anti-DNP plaque forming cells, and the results are expressed as direct and indirect PFC per 10^6 viable lymphocytes.

d) Results represent geometric means ± standard errors.

Table II

GENETIC CONTROL OF LYMPH NODE RESPONSE TO DNP-Pro_{22}-Tyr-ABA

	SJL ($H\text{-}2^s$)		DBA/1 ($H\text{-}2^q$)		SWR ($H\text{-}2^q$)	
dpi[a]	IgM[b]	IgG	IgM	IgG	IgM	IgG
7	0	0	0	0	0	0
9	N.D.[c]	N.D.	N.D.	N.D.	N.D.	N.D.
12	0	12±5	0	<1	0	<1
14	<1	10±2	0	2±1	0	1±1
16	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18	0	2±1	0	0	0	<1

	CBA/J ($H\text{-}2^k$)		C57Bl/6 ($H\text{-}2^b$)		A/J ($H\text{-}2^a$)	
dpi	IgM	IgG	IgM	IgG	IgM	IgG
9	<1	0	1	<1	3	0
12	2±.4	2±.2	4±2	14±31	9±5	32±40
14	1±.4	3±1	2±1	38±14	3±1	99±10
16	2±1	4±3	1±.3	5±3	6±4	137±205
18	<1	<1	1±.2	11±3	3±1	6±3
20	<1	<1	<1	5±.5	<1	48±42

a) dpi=days post immunization

b) 100 µgr of antigen in CFA is injected subcutaneously into the hind footpads,and the draining popliteal and inguinal lymph nodes are assayed for anti-DNP plaque forming cells. The results are expressed as IgM- or IgG-PFC per 10^6 viable lymphocytes.CFA control values are either 0 or less than 1.

c) N.D.=not determined

Table III

RESPONSE OF (Balb/c,♀ x CBA/N,♂) F_1 MICE TO TI-1(TNP-LPS), TI-2(DNP-Ficoll) AND TD(DNP-OVA) ANTIGENS (footpad injected)

		TNP-LPS[a]				DNP-Ficoll[b]				DNP-OVA[c]			
		♀		♂		♀		♂		♀		♂	
Organ	dpi	IgM[f]	IgG[g]	IgM	IgG	IgM	IgG	IgM	IgM	IgM	IgG	IgM	IgG
Spl.[d]	4	34	<1	ND[h]	ND	168	12	ND	ND	ND	ND	ND	ND
	6	34	0	66	2	122	6	151	20	33	1	26	0
		37	0	44	0	112	2	179	40	ND	ND	ND	ND
	7	28	0	21	0	129	20	76	5	33	10	20	3
		21	0	31	0	174	20	224	23	25	9	36	4
	10	25	0	39	1	1	2	1	3	37	25	43	10
	12	33	0	41	3	81	1	ND	ND	31	29	19	18
										24	14		
	14									26	19	16	13
L.N.[e]	4	<1	0	ND	ND	13	1	ND	ND	ND	ND	ND	ND
	6	5	0	7	0	19	15	75	14	29	459	46	198
		0	0	2	0	7	5	21	31	ND	ND	ND	ND
	7	0	0	0	0	27	64	22	35	57	2023	91	3617
		0	0	0	0	12	15	28	115	23	1266	34	945
	10	2	1	0	0	1	166	3	1	79	2467	73	1180
	12	1	0	1	0	2	<1	5	3	216	956	33	160
										99	534		
	14									27	610	9	240

a,b) 10 µgr of antigen is used per mouse

c) 100 µgr of antigen is used per mouse

d) Spleen.CFA control values: 10-20 IgM-PFC/10^6, 0 IgG-PFC

e) Lymph nodes.CFA control values:<1 IgM-PFC/10^6,0 IgG-PFC

f,g) IgM and IgG plaque forming cells per 10^6 lymphocytes

Table IV

EFFECT OF CYCLOPHOSPHAMIDE(CY) TREATMENT ON THE SPLENIC RESPONSE TO BIFUNCTIONAL ANTIGENS

A) 20 mg CY / kg of body weight , 1 day before antigenic challenge

dpi	CY treatment	IgM-PFC/10^6cells[a]
8	-	53
	+	65
11	-	38
	+	22
14	-	11
	+	55
18	-	72
	+	25
21	-	32
	+	27

a) 100 µgr of DNP-sac-Pro_{22}-Tyr-ABA was used per mouse. IgG-PFC/10^6 values were less than 1.

B) 100 mg CY / kg of body weight , 2 days before antigenic challenge

	IgM-PFC/10^6cells[a] induced with	
dpi	DNP-sac-Tyr-ABA(ip)	DNP-Pro_{22}-Tyr-ABA(ip)
10	20±15	15±1
17	26±2	27±3
20	40±3	25±12

a) IgG-PFC/10^6 values were less than 1

Table V

TIME COURSE STUDY OF THE SUPPRESSION INDUCED WITH THE BIFUNCTIONAL ANTIGENS (i.p.)

dpi	Time interval	IgG-PFC/10^6 anti-DNP responses[a]	anti-BSA responses[b]
11	control[c]	197±23	321±127
	1 week	10±2	
	3 weeks	3±1	265±20
	14 weeks	241±65	
14	control	41±1	268±45
	1 week	1±.4	
	3 weeks	4±1	177±31
	14 weeks	57±22	
17	control	14±3	325±32
	1 week	<1	
	3 weeks	1±.2	241±86
	14 weeks	4±1	

a) Mice were injected with either DNP-sac-Tyr-ABA (i.p.) or CFA (control),and 1, 3 or 14 weeks later,they were challenged with DNP-Pro_{22}-Tyr-ABA in the footpads.Popliteal and inguinal lymph nodes were assayed for anti-DNP PFC.

b) Some groups of mice were challenged with BSA (f.p.),and anti-BSA IgG-PFC/10^6 were assayed.

c) Control mice were injected with Complete Freund's adjuvant.

Table VI

PANNING OF SUPPRESSOR CELLS ON ANTIGEN COATED PLATES

Cells transferred[a]	IgG-PFC/10^6cells[b]	% suppression
B cells and plastic adhering cells[c]	260±42	-
ABA-adherent T cells	8±3	97
ABA-nonadherent T cells	59±7	77
DNP-adherent T cells	36±6	86
DNP-nonadherent T cells	38±6	85
CFA primed cells	253±64[d]	-
T suppressor cells	119±39	53

a) Donor mice were injected with 100 µgr of DNP-sac-Tyr-ABA i.p. 2 weeks before the cell transfer.

b) Recipient mice were primed with 100 µgr of DNP-Pro_{22}-Tyr-ABA, f.p., 1 day before the cell transfer.

c) Spleen cells were first adsorbed on anti-mouse immunoglobulin coated plates,the bound B cells and plastic adhering cells were retrieved and transferred separately.The non-adhered T cells were then panned on either on ABA- or DNP-coated plates.Antigen binding and notbinding cells were recovered and transferred.

d) The recipient mice in this group were challenged with DNP-BSA 1 day before the cell transfer.

Table VII

PRELIMINARY IDIOTYPIC ANALYSIS OF THE RESPONSE

A) Suppression of anti-DNP response

	Suppression induced with			
	DNP-sac-Tyr-ABA (i.p.)		Tyr-ABA (i.p.)	
	% suppression of		% suppression of	
dpi[a]	IgM-PFC	IgG-PFC[b]	IgM-PFC	IgG-PFC
6	54	56	44	43
8	17	52	21	42
12	29	33	40	27
15	8	28	76	43

a) dpi=days post immunization

b) 2 weeks after the i.p. immunization,mice were challenged with DNP-OVA in the footpads and the popliteal and inguinal lymph nodes were assayed for anti-DNP plaques.

B) Plaque inhibition with rabbit anti-CRI[c]

Suppression induction(i.p.)	Antigenic challenge(f.p.)	%inhibition of plaques	mean
CFA	DNP-OVA	22,24,22,22,55,43[a]	29
DNP-sac-Tyr-ABA	DNP-OVA	2,0,0,0,0,1[a]	<1
Tyr-ABA	DNP-OVA	2,0,0,3,0,1[a]	<1
CFA	ABA-BSA	32,35,42,29,58,54,57,7[b]	34
DNP-sac-Tyr-ABA	ABA-BSA	25,76,35,23,63,76,41,16[b]	38

a) anti-DNP plaques were developed

b) anti-ABA plaques were developed

C) Plaque inhibition with anti-MOPC-460 and anti-CRI[c]

		%inhibition of plaques with[a]	
1st challenge(ip)	2nd challenge(fp)	anti-CRI[c]	anti-MOPC-460
CFA	DNP-Pro_{22}-Tyr-ABA	39,24,27	51,39,40
DNP-sac-Tyr-ABA	"	- not enough numbers of	
Tyr-ABA	"	plaques to analyze-	
DNP-Lys	"	28,16,13	48,39,44

a) Inhibition of anti-DNP plaques on days 9,11,13.