SUPPRESSION OF REAGINIC ANTIBODY PRODUCTION WITH POLYVINYL ALCOHOL AS TOLEROGENIC CARRIER

by

Dawn A. Hubbard, B.Sc. (Honours)

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of Master of Science.

Department of Immunology,
Faculty of Medicine,
University of Manitoba,
Winnipeg, Manitoba,
R3E 0W3.

August, 1979.

SUPPRESSION OF REAGINIC ANTIBODY PRODUCTION WITH POLYVINYL ALCOHOL AS TOLEROGENIC CARRIER

BY

DAWN ARLENE HUBBARD

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

SUPPRESSION OF REAGINIC ANTIBODY PRODUCTION WITH POLYVINYL ALCOHOL AS TOLEROGENIC CARRIER

ABSTRACT

Conjugates of polyvinyl alcohol, a synthetic water-soluble polymer of molecular weights 10,000 and 14,000 daltons, and two hapten molecules, DNP and BPO, were prepared and the effect of these conjugates on the IgE and IgG responses of BDF_1 mice was studied. Pre-administration of a single, low dose of either DNP-PVA or BPO-PVA, was sufficient to suppress completely a primary anti-hapten IgE response. In addition, treatment of sensitized mice with the conjugates resulted in an abrogation of the ongoing anti-hapten IgE response. The anti-DNP hemagglutinating antibody response was suppressed also by these treatments, but at no time was the anti-carrier antibody response, either IgE or IgG, affected by these tolerizations.

The epitope density of the hapten on the PVA molecule was found to play an important role in the establishment of an unresponsive state and in the allergenicity of the conjugate. Conjugates with epitope densities of less than 1.0 were able to suppress an anti-hapten IgE response, but complete and long-lasting suppression of the reaginic response was not realized unless a conjugate of epitope density greater than 1.0 was employed. However, the conjugates with lower epitope densities had lower allergenicity.

In addition, mice immunized with the sensitizing dose of antigen plus Bordetella pertussis, were sensitive to anaphylaxis upon intravenous challenge with the multivalent hapten-protein conjugate. However, mice that received a tolerizing dose of

either DNP-PVA or BPO-PVA, were protected from anaphylaxis induced by the appropriate hapten-protein conjugate. The protection afforded by these compounds is highly specific for the hapten and anaphylaxis occurred in the protected animals upon challenge with the carrier protein.

As well, preliminary adoptive cell transfer experiments demonstrated that the unresponsive state could be transferred into X-irradiated syngeneic recipients and the suppression was at least partially mediated by suppressor cells.

ACKNOWLEDGEMENTS

I would like to express my sincerest appreciation to Dr. Weng Y. Lee for the guidance and assistance that he has given me during my M.Sc. program. His supervision and help have been invaluable during this time.

I would also like to thank all the members of Dr. Lee's laboratory, especially Valerie Cripps and Lionel Filion, for their friendship and support in my work.

Finally, I would like to acknowledge the excellent technical assistance of Ms. V.J. Cripps and MS. K.M. Nawrocki in performing the experiments listed in Tables XI and XVIII and Figure 3.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A/J	- inbred strain of mouse
AKR	- inbred strain of mouse
Asc	- *Ascaris suum* extract
BALB/c	- inbred strain of mouse
B cell	- lymphocyte derived from bone marrow
BDF_1	- inbred strain of mouse; $(C57BL/6 \times DBA/2)F_1$
B_ε cell	- B-cell producing IgE antibodies
BγG	- bovine gamma globulin
BPO	- benzylpenicilloyl determinant
C57BL/6J	- inbred strain of mouse
CMC	- carboxymethyl cellulose
cm	- centimeter
D-GL	- random copolymer of D-glutamic acid and D-lysine
DNP	- 2,4-dinitrophenyl determinant
ECF-A	- eosinophil chemotactic factor of anaphylaxis
g	- gram
H-2	- major histocompatibility complex of the mouse
HA	- passive hemagglutination test
HSA	- human serum albumin
IgE	- immunoglobulin of class E
IgG	- immunoglobulin of class G
IgM	- immunoglobulin of class M
i.d.	- intradermally
i.p.	- intraperitoneally
i.v.	- intravenous
KLH	- keyhole limpet hemocyanin
LE	- levan

MHC	- major histocompatibility complex
µg	- microgram
µl	- microliter
mg	- milligram
ml	- milliliter
mm	- millimeter
mM	- millimolar
M	- molar
MγG	- mouse gamma globulin
NIP	- 4-hydroxy-3-iodo-5-nitrophenacetyl determinant
NRS	- normal rabbit serum
OA	- ovalbumin
PAB	- para-aminobenzoic acid
PCA	- passive cutaneous anaphylaxis
PEG	- polyethylene glycol
PFC	- plaque forming cells
PVA	- polyvinyl alcohol
PVP	- poly(N-pyrrolidone)
R	- rads of radiation
RAG	- non-dialyzable aqueous extract of ragweed pollen
SJL	- inbred strain of mouse
SRBC	- sheep red blood cells
SRS-A	- slow reacting substance of anaphylaxis
T cell	- lymphocyte derived from the thymus
Tbc	- Mycobacterium
Θ	- 'theta' antigen present on T lymphocytes
TsF	- T-cell suppressor factor
UD	- urea-denatured

INTRODUCTION

Hay fever is a representative of human atopic allergies that has plagued man for centuries. Although it is usually not lethal or greatly debilitating, it, as well as other miriad of atopic allergies, inflicts upon millions of people pain and inconvenience and in some instances, the constant fear of anaphylaxis upon exposure to the allergen. Consequently, ever since hypersensitivity has been recognized as a response of the immune system, medical researchers and medical doctors have been searching for a safe, guaranteed treatment, a cure and hopefully a prevention.

Atopic allergies, hay fever amongst them, were first recognized as immunologic phenomena in the early 1900's. In 1902, Richet and Portier observed anaphylaxis in dogs upon a second injection of a glycerin extract of the tentacles of Actinaria. The first injection of a small dose produced no detectable response but the supposedly protected animals suffered shock, usually lethal, following the second injection (1). Following this work, there was a number of findings concerning hypersensitivity reactions, all indicating the involvement of the immune system in the development of a hypersensitive reaction. These include:

1. the description of the Arthus phenomenon by Arthus. This is the production of a local necrotic lesion by a secondary immunization of an antigen;
2. studies on serum sickness, following a secondary injection of antistreptococcal serum by von Pirquet and Schick (1905);

3. work by Theobald Smith and Otto (1906) as well as Rosenau and Anderson (1909), that showed that: a) the secondary reaction to diptheria toxin and antiserum in guinea pigs was due to antibodies against the antiserum; b) the time of sensitization was ten days; and c) a secondary reaction could be produced by passive sensitization with serum from a previously sensitized animal;

4. Prausnitz and Kustner (1921) proved the concept of hay fever as a hypersensitivity reaction (1).

With the identification of IgE as the reaginic antibody responsible for the hypersensitivity, a giant step was taken in allergy research. Since that time, a great deal of knowledge about allergies and the allergic response has accumulated. The allergic response involves the interaction and the co-ordination of many components of the immune system, and the control of this response involves even more components of this system.

The majority of the symptoms of the immediate hypersensitive reaction are a result of the action of histamine. The histamine is released from mast cells or basophils, following degranulation of the same cells after the cross-linking of the IgE molecules by the allergen on the surface of the mast cell or basophil. Consequently, in an effort to control the symptoms of atopic allergies, anti-histamines have been widely used. Anti-histamines function in blocking the receptors of the histamines released during degranulation. As a result of prior treatment with anti-histamine, on exposure to the allergen and the concomittant release of histamine,

symptoms are reduced because the histamine receptors on the shock organs are already occupied by the anti-histamines. This protocol does not however relieve all of the symptoms because there are several other pharmacologically active substances that are also released during degranulation, including SRS-A, eosinophil chemotactic factor of anaphylaxis(ECF-A), and bradykinin. The action of these agents is of course not affected by the use of anti-histamines.

Desensitization by the repeated injections of allergen has also been used extensively for a number of years in the treatment of atopic allergy. This technique is designed to increase the quantity of blocking antibodies of the IgG class in the system, such that upon exposure to the allergen, the IgG will remove the allergen from circulation. If the allergen can not react with the IgE on the target cells, there will be no hypersensitive reaction. This procedure has had a long but unpredictable history. In some patients, hyposensitization results in a marked improvement in symptoms, but in others, there is no effect (1-2).

Both of these treatments and others that have been used to treat the atopic allergic patient are aimed at relieving the symptoms and can not be regarded as a cure. Consequently, in recent years, attention has been turned to the cellular events in the establishment of an allergic response in the hopes of determining a protocol that could be used to eliminate an ongoing allergic response or prevent the production of such a response.

Recently, much attention has been focused upon the inter-

action between T cells and B cells in the initiation of an IgE response. The desire has been to find a means of interfering in this collaboration between the cells so that the IgE response can be controlled.

Claman was the first to demonstrate directly, that two distinct cell populations,bone marrow (B) lymphocytes and thymus-derived (T) lymphocytes must act collaboratively in the initiation of an antibody response to certain antigens, such as sheep erythrocytes and protein antigens (3). These workers demonstrated that before an anti-SRBC response could be developed in lethally irradiated animals, both syngeneic thymus and bone marrow cells must be transferred into the animals. As well, it was shown that sonicated or irradiated thymus cells were not competent in raising an antibody response and thus living syngeneic thymus cells are required. Comparable results have been found by Davies (4-5), who also found that T lymphocytes proliferate upon antigenic stimulation (6) and that these thymus-derived cells were not responsible for the synthesis of antibody following such an antigenic stimulation (7).

Further evidence supporting the theory of necessary collaboration between B and T cells in the production of an antibody response was provided by the work of Miller, Mitchell and their associates (8). These workers found that the transfer of syngeneic thymus cells or thoracic duct lymphocytes restored the ability of neonatally thymectomized mice to respond to SRBC and that cells of the recipients were actually producing the anti-SRBC antibodies (9-10). Specifically, it

was found that the cells that were synthesizing the antibodies were from the bone marrow-derived lymphocyte population, i.e. B cells. Similar observations of concomittant T-B cell collaboration in an immune response have been shown for protein antigens (11-13) as well as for protein carrier-hapten antigen conjugates (14-20).

The work cited thus far clearly demonstrated the requirement of T-B cell interaction in an IgM and IgG immune response. A considerable amount of work has been published that also supports the concept of T-B cell collaboration in the IgE immune response (21-27). The most convincing work comes from the laboratory of Hamaoka and his coworkers (27). These researchers, using an adoptive cell transfer technique, have shown clearly that carrier-specific helper cells for the IgE antibody response are Θ-bearing T lymphocytes. The researchers found that an IgE response was only developed in irradiated recipients when cells primed with the appropriate carrier were transferred into animals, along with cells primed to the hapten attached to a different protein carrier. The helper function supplied by these carrier-primed cells was abolished with treatment of the cells with anti-Θ antiserum plus complement. Similar supporting evidence has been reported by other workers (21-26).

At about this time as well, much more work was being done in the area of tolerance induction in the IgG and IgM responses. In 1969, it had been established by Havas (28) that when using hapten-protein conjugates, tolerance to the hapten was developed if the carrier had low immunogenicity, whereas an immune response could easily be established against

the carrier and the hapten, if the carrier was itself immunogenic. Thus several studies were conducted using this principal.

Golan and Borel (29) established a state of unresponsiveness to DNP, by injecting conjugates of DNP and isogenic gamma globulin. As well, conjugates of haptens and syngeneic erythrocytes have been used by Hamilton and Miller (30) to establish a state of unresponsiveness.

In the latter study, the immune response to the hapten, as determined by plaque forming cells (PFC), was greatly reduced with pretreatment of the mice with a conjugate of the hapten with syngeneic erythrocytes. Both the primary and secondary responses of the animals were found to be suppressed and as well, the unresponsiveness could be serially transferred into irradiated recipients. By using cell transfer studies, these researchers came to the conclusion that the unresponsiveness of the treated mice was a result of a lesion in the T cell depleted spleen cell population, i.e. in the B cell population (31). Futhermore, a hapten-specific unresponsive state in the IgM and IgG producing B cells had been established through treatment with the non-immunogneic DNP-conjugate of a copolymer of D-glutamic acid and D-lysine (D-GL) (32-33).

The knowledge that had been gained by the workers investigating tolerance in the IgM and IgG responses was now applied by the workers examining the reaginic response. With the new understanding of the IgE response in terms of the collaboration between B and T cells, (paralleling the situation in the IgM and the IgG responses), researchers began to investigate the phenomenon of tolerance in the hopes that methods used in the

IgM and IgG systems would be applicable. The first work along these lines was done by Katz et al. (34). Using conjugates of D-GL and conditions similar to those that produced tolerance in the IgG and IgM responses (32, 34) they were able to establish a hapten-specific unresponsive state in IgE producing cells. The workers demonstrated the establishment of a tolerant state in both virgin and primed A/J mice using cell transfer techniques. The tolerant state could not be reversed by treating the cells with trypsin (32) indicating that cell reactivity to DNP determinants was irreversibly inhibited.

In a similar system developed by Lee and Sehon (60) (to be discussed in depth later), tolerance in the IgE B cell population was also developed using DNP- or BPO-conjugated isologous γ-globulins.

It seems that tolerance can be easily developed using this type of protocol. However, an unresponsive state in the IgE producing cells can not explain all states of tolerance that have been established and examined. Because of the important role played by T cells in the initiation of an IgE response, it is also reasonable to expect T cells to play an important role in the induction of a tolerant state. In a number of instances, it has been found that the depletion of T cells in an animal enhances the IgE response. Okumura and Tada (35) have shown that adult thymectomy or splenectomy of rats greatly enhanced the IgE response in these animals. A similar result was found in mice by Michael and Bernstein (36-37). These workers found that reconstituting nude mice with a small number of thymocytes ($5x10^5$ cells) produced a persistent antibody

response of the IgE class, while reconstitution with a higher number ($5x10^7$ cells) resulted in an earlier termination of the antibody response. An IgM/IgG antibody response to SRBC did not occur until a higher number of thymocytes was used in the reconstitution and with this number of thymocytes, the IgE response was again suppressed.

Recently, Watanabe et al. (38) and Chiorazzi et al. (39-40) have explained these and other similar findings with their evidences supporting the existence of non-specific suppressor T cells which regulate IgE antibody responses. These workers showed that treatment of carrier primed mice with low dose irradiation or cyclophosphamide enhanced IgE anti-hapten antibody response upon immunization with hapten-homologous carrier conjugates. The IgG response was unaffected and the enhancement was abolished with the transfer of normal spleen cells or thymocytes into the irradiated mice (38,40). Pretreatment of unprimed low responder (AKR) and non-responder (SJL) mice with X-rays, cyclophosphamide or an adequate dose of anti-lymphocyte serum, enhanced the IgE response to subsequent antigen immunization (40).

To explain these results, the investigators suggested that low or non-responder strains of mice were not incapable of mounting an IgE response, but rather the lack of a response was due to the presence of non-antigen-specific T cells which were actively suppressing a response. These suppressor T cells were acting to control the IgE response at one or more levels of control:

1. at the level of induction of carrier specific helper T-cells;

or 2. by inhibition of T-B cell co-operation or suppression of B cell differentiation or of its function directly (40).

The existence of specific suppressor T cells has also been demonstrated, which cause a suppression of the IgE response (41). Ishizaka and his coworkers have used urea-denatured ovalbumin (UD-OA) to tolerize BDF_1 mice to native ovalbumin. These investigators found that intravenous injections of UD-OA into OA primed animals not only suppressed the response to OA, but also to DNP-OA upon subsequent immunization. To further clarify the characteristics of the suppression, the researchers supplemented UD-OA treated animals and normal animals with OA primed spleen cells. The response of the normal animals was augmented upon supplementation with the OA primed spleen cells. However, there was no increase in response of the UD-OA treated animals upon immunization with DNP-OA. Thus, the results indicated that the suppression of the response is an active process. Also, the transfer of UD-OA splenic T cells into normal non-irradiated animals greatly decreased the antibody response of the recipients to DNP-OA.

These suppressor T cells were specific for the carrier OA, since a normal anti-DNP response could be generated following immunization with DNP-KLH. The effect of these suppressor T cells on an ongoing IgE antibody response was also investigated by Ishizaka *et al.* (42). Splenic T cells from UD-OA treated animals were transferred into animals displaying an IgE response to DNP-OA and the IgE titer of these animals was followed. These suppressor T cells depressed the ongoing

antibody response, whereas transfer of OA primed splenic T cells failed to depress the antibody formation. Thus, these suppressor T cells were found to be able to suppress not only a primary IgE antibody response, but also an ongoing IgE antibody response.

In other experiments, these workers found that the suppressive effect of the UD-OA treated splenic T cells could be enhanced by co-culturing with OA or OA bearing macrophage prior to transfer into irradiated recipients. These results lend support to the concept of macrophage involvement in the establishment of a tolerant state induced by T cells (43-44).

Further information concerning suppressor T cells has been gained from the work of Tada and others, working with the rat. A reaginic response is difficult to establish and maintain in the rat, unlike most mouse species and the human. As a result, the rat system offers a unique situation for the investigation of factors causing early termination of a reaginic response (45).

The first indication that the antigen-specific suppressor T cell was involved in the normal rat reaginic response came with the observation that treatment of the rat with a number of immunosuppressive agents resulted in an enhancement of the IgE response. Tada and his coworkers found a number of methods such as sublethal X-irradiation, adult thymectomy and splenectomy and treatment with immunosuppressive chemicals such as 5-bromodeoxyuridine, cyclophosphamide, actinomycin D, and anti-thymocyte serum, which decrease the T cell population, resulted in an enhanced IgE response (35, 46-49).

Further support for the suppressor T cell as the cause of the early termination of the reaginic response was found in cell transfer studies done by Tada and his collaborators (50). In this work, it was found that a responsive state, (established by using sublethal X-irradiation) could only be abolished by the transfer of thymocytes from rats hyperimmunized with the same carrier or carrier conjugate in complete Freund's adjuvant. Cells from normal animals or animals hyperimmunized with a different carrier had no effect on the reaginic response. Because the inhibition was carrier specific, and a direct result of the thymocytes themselves, the researchers suggested that T cells stimulated by carrier determinants suppressed the ongoing reaginic antibody synthesis of the recipient and that the immunosuppressive treatments previously described, interfere in this regulatory mechanism, resulting in the enhanced and sustained production of reaginic antibody.

Tada and his coworkers have further been able to characterize the soluble factor that is responsible for the suppressive activity of the T cells (51-52). The following properties of the factor have been elucidated:

a) the factor is carrier specific in its ability to suppress a response due to its specific binding affinity for the carrier determinants;

b) the factor is not an immunoglobulin;

c) the factor originates in the T cell membrane;

d) the factor is not a complex containing antigen;

e) the factor is not destroyed by digestion with ribonuclease

or deoxyribonuclease;

f) the factor is quickly inactivated by digestion with trypsin or pronase;

g) the molecular weight of the factor is estimated to be between 35,000 and 60,000 daltons by preparative ultracentrifugation and gel-filtration with Sephadex G-200;

h) the electrophorectic mobility of the factor is β to α.

These initial studies on the rat prompted Tada to look at the suppression of IgG responses, that they had been able to establish in inbred mice. They were able to extract a soluble factor from suppressor T cells and it was found to have similar characteristics as the factor found in the rat system (53-55). Continuing with this work and using inbred strains of mice that differed in H-2 haplotypes, Tada and his coworkers established the genetic requirements for the interaction of the suppressor and acceptor cells. With the use of alloantisera to various subregions of the H-2 complex, the T cell factor was found to be a product of a restricted subregion. This led to the definition of a new subregion (I-J) in the I region of the H-2 complex (56-57). This new region was defined by the presence of a gene or genes which code for a unique molecule on the suppressor T cell surface.

In further studies (58-59), Tada clearly demonstrated that:

a) alloantisera against the I region of the H-2 complex absorbed the suppressive T cell factor;

b) the factor could not be absorbed by antisera against histocompatibility antigens coded by the K or D regions;

c) anti-Ia specificities, which had been determined by cytotoxic activity for B cells did not correlate with the absorbing power of the alloantisera;

d) absorbing antisera always had specificity against the I-J subregion, whereas antisera lacking this specificity also lacked the absorbing power;

e) the suppressive factor obtained from one strain of mouse could effectively suppress the response in another strain sharing the same I-J subregion; and

f) the acceptor site for the T cell suppressor factor was postulated to be coded for by the genes in the same I-J subregion.

Consequently, it has been suggested that the I-J subregion genes code for the synthesis of complementary surface molecules on two separate and distinct subsets of lymphoid cells, through which the suppressive interaction can be achieved.

In another system, Lee and Sehon have examined the effects of several different tolerogens on the IgE response. These researchers have found that treatment of BDF_1 mice with hapten-isologous-γ-globulin conjugates is effective in suppressing the anti-hapten IgE response. The initial work was done using DNP-MγG (60-61) and it was found that an intravenous injection of DNP-MγG could prevent a primary reaginic response in normal animals and also abrogate an ongoing anti-DNP response. The tolerant state of these animals could be maintained for an extended period of time (experimentally determined up to eight months) by repeated injections of the tolerogen at periods of two months. Using the same system, cell transfer work

was done to determine the nature of the suppression and to determine if the suppression was simply due to the neutralization of antibody by the tolerogen. The simple neutralization of the antibody could not explain the long term tolerant state in the mice, as the tolerogen would be cleared from the animal in approximately thirty days. However, to gain further supportive evidence for the establishment of a true unresponsive state, adoptive cell tranfer studies in syngeneic mice were performed.

Several adoptive transfers were performed and all supported the establishment of a true tolerant state. The transfer of cells, from animals tolerized 51 days prior, into X-irradiated animals resulted in the maintenance of a tolerant state in the recipient animals after a sensitizing dose of antigen was administered. Tolerance could not be established using the serum of tolerized animals, tolerized 20 days prior to bleeding, indicating that the tolerogen had been cleared by this time. The unresponsive state of the mice could not be maintained, however, with injection of normal or primed cells into the tolerant animals, but rather these animals responded according to the type of cell they received (i.e. either a primary or a secondary response was exhibited). Similarly, an admixture of tolerant and primed or normal cells administered into X-irradiated recipients did not result in tolerance, but instead a secondary or primary response was demonstrated. This further supports the assumption that the tolerogen has been cleared from the animal and that the unresponsive state is definitely the result of the initial encounter of the

immunocompetent cells with the tolerogen. Maintenance of the tolerant state in repeated exposure to antigenic stimulation in two successive transfers into irradiated recipients is further evidence supporting this conclusion.

Further, Lee and Sehon found evidence that particularly implicated the inactivation or elimination of the B_{ε} cell population as the cause of the tolerant state developed in this system (62). The evidence supporting this hypothesis is as follows:

1. an antibody response was produced in X-irradiated recipients upon transfer of thymus cells from tolerant animals and primed bone marrow cells;
2. no antibody response was detected in the X-irradiated recipients after transfer of a mixture of tolerant bone marrow cells and primed thymus cells;
3. an antibody response was produced in X-irradiated recipients when a mixture of tolerant spleen cells and primed bone marrow cells were transferred;
4. an antibody response could not be restored by supplementing a transfer of tolerant spleen cells into X-irradiated recipients with an excess of thymus cells from primed mice.

The isologous-γ- globulin system was also extended by Lee and Sehon in the examination of the penicillin system(63). Tolerogenic conjugates of MγG and penicillin G were prepared and used to establish tolerant states in BDF_1 mice. The results obtained in this system were virtually identical to those obtained in the DNP-MγG system previously discussed. The tolerant state could be established in normal and primed animals

and could be transferred into X-irradiated recipients with the transfer of tolerant spleen cells. Also, as in the DNP system, the tolerant state was specific for the hapten conjugated to the MγG and the response of the animals to the carrier OA was not affected.

Further to the use of the MγG system to establish an unresponsive state to a hapten group, other work has been completed in this laboratory using the MγG system to establish tolerance to a protein carrier molecule (64). Conjugates of MγG and ovalbumin (OA) were prepared with varying compositions ($(OA)_{12}$ - $(M\gamma G)_2$ being the average) and were used to produce a state of unresponsiveness in BDF_1 mice. Tolerization with OA-MγG resulted in markedly reduced antibody production to the OA carrier or to DNP when DNP-OA was the sensitizing antigen. However, anti-DNP antibody could be produced if DNP-Asc in the presence of $Al(OH)_3$ was used as the sensitizing antigen. Treatment of the animals with OA-MγG suppressed both the primary and the secondary responses of the animals specifically to OA. In an attempt to elucidate the possible mechanism(s) of tolerance induction in this system, adoptive cell transfer work was performed. The transfer of cells from tolerant animals into intact, normal recipients greatly inhibited the ability of the recipients to respond to DNP-OA. This result indicates the generation of suppressor cells upon treatment with OA-MγG. This is also supported by the fact that tolerance could not be broken by transferring normal or DNP-OA primed cells into tolerant recipients.

These results suggest the generation of suppressor cells in the system, which is contrary to the evidence found in the DNP-MγG system. Thus, the mode of action must be somewhat different for the MγG-protein conjugates as compared to the MγG-hapten conjugates, in the establishment of a state of tolerance.

Another system of tolerance induction using polyethylene glycol (PEG) has also been developed in this laboratory (65-66). Conjugates of PEG and OA or with the nondialysable constituents of the aqueous extract of ragweed pollen (RAG) were prepared and used to tolerize BDF_1 mice. In the studies, it was found that OA-PEG could suppress both a primary and a secondary response to the sensitizing antigen DNP_3-OA in $Al(OH)_3$. It was also found that the conjugates themselves were devoid of the ability to combine with antibody induced to the unmodified antigen; either in vivo or in vitro. Thus, the conjugates could not elicit a passive cutaneous anaphylaxis (PCA) reaction at sites previously sensitized with murine IgE, nor could they cause systemic anaphylaxis in rats sensitized to OA or combine with anti-OA serum to form precipitin bands. As well, the slow clearance of the OA-PEG conjugates as compared to that of native OA, support the evidence for nonimmunogenicity of the PEG conjugates. The nonimmunogenicity of the conjugates and their tolerogenicity raises the possibility of their use in the clinical desensitization treatment of human patients (65).

Cell transfer work was also done in this system in an attempt to identify the mode of action of the PEG conjugates.

The state of tolerance could be maintained by transfer of tolerized spleen cells into X-irradiated recipients. Also, preliminary results showed that transfer of tolerized spleen cells into normal animals led to suppressed anti-OA and anti-DNP responses following sensitization with DNP_3-OA in $Al(OH)_3$ indicating the presence of a suppressor cell(66).

In another system of great interest, Kishimoto and his coworkers have extensively investigated the effect of DNP-coupled Mycobacterium on the IgE and IgG responses of mice (67). The initial work of these researchers indicated a very strong suppressive effect of the DNP-coupled Mycobacterium (DNP-Tbc) on the IgE anti-DNP and anti-OA response of animals pretreated with DNP-Tbc and then challenged with DNP-OA in alum. At the same time, however, the anti-DNP IgG response was unaffected. Similar results were obtained in the in vitro culture of DNP-OA primed mouse spleen cells with DNP-Tbc primed spleen cells. The anti-DNP IgE response was completely suppressed, while the anti-DNP IgG response was unaffected by the addition of the DNP-Tbc primed cells.

Further investigation using a cell transfer system, revealed that the transfer of DNP-Tbc primed spleen cells into normal syngeneic recipients greatly suppressed the anti-DNP IgE response but did not influence the anti-DNP IgG response of these animals. As well, the workers transferred the B cell depleted fraction of DNP-Tbc primed spleen cells into normal mice and again found the suppressive effect on the anti-DNP IgE response, but not on the IgG response. Similar results were also obtained in the in vitro culture of DNP-OA primed

spleen cells with the B cell depleted fraction of DNP-Tbc primed spleen cells. Thus the results strongly indicated the presence of a suppressor T cell in the B cell depleted DNP-Tbc primed fraction that specifically suppresses the IgE response while not influencing the IgG response.

Continuing the work (68) in this system, Kishimoto et al. have found that it is indeed a T cell that is responsible for the suppression of anti-DNP IgE response resulting from the treatment with DNP-Tbc. In the in vitro culture system, it was shown that the suppressive effect of the DNP-Tbc primed spleen cells was eliminated following treatment with anti-θ and complement, but was not diminished by the depletion of B cells by anti-Ig column. The IgE class specific suppressor activity of the DNP-Tbc primed T cells was also eliminated with X-irradiation. As well, it was established that the suppressive effect on the anti-DNP IgE response was mediated by a soluble factor produced by the DNP-Tbc primed T cells. Interestingly, it was also found that this factor did not possess any antigen specificity. Thus, the suppressive effect of the cell free supernatant was not decreased following absorption with DNP-HSA, even though specific antigen stimulation at the cellular level was required before the suppressive activity was produced. The suppressive activity of the DNP-Tbc primed cells was only produced with stimulation by DNP-OA not with PAB-OA (PAB=para-aminobenzoic acid), but once the activity was generated, it suppressed the IgE response to both DNP and OA.

Having isolated the soluble suppressive factor from the DNP-Tbc primed T cells, Kishimoto et al. did further work to

characterize the factor (69). A combination of *in vitro* and *in vivo* systems was used in this work and it was found that the IgE class specific suppressor factor (IgE-TsF) had gene products of the major histocompatibility complex (MHC). The precise locus was not located, but the suppressor activity of the IgE-TsF was absorbed by anti-Ia antiserum specific for the I-A to I-E regions. As well, MHC homology was found to be a requirement before the suppressor factor was active. Consequently, IgE-TsF released from BALB/c T cells failed to suppress the IgE response of C57BL/6J mice while the factor obtained from C57BL/6J cells was active in C57BL/6J animals, but not in BALB/c mice.

The target cells for the IgE-TsF were found to be B cells committed for the IgE antibody response, and thus had to be stimulated by DNP-OA before they would absorb the suppressor activity. Once stimulated with DNP-OA, the suppressor activity was only displayed when IgE-TsF was added to the B cells, which were no longer available to synthesize anti-DNP IgE antibodies.

To continue the investigation of the properties and functions of the IgE-TsF, these workers attempted to establish a T hybrid cell line which secreted the class specific IgE suppressor factor (70). The successful establishment of such a cell line secreting suppressor factor(s), which appeared to have the same characteristics of the IgE-TsF produced by DNP-Tbc primed T cells, will allow the authors to further characterize the nature of this suppressive factor.

Several other workers have investigated various systems

in which a hapten is conjugated to a high molecular weight carrier and this conjugate is used to initiate an unresponsive state in the animal. Some of the carriers used are derived from natural sources, such as Mycobacterium tuberculosis, used by Kishimoto (67-70) or the mouse gamma globulin used by Lee (60-64). As well, levan conjugates have been used by Desaymard and others (71-72) to induce tolerance to various haptens. The high molecular weight levan is isolated from Corynebacterium levaniformis and is conjugated to the hapten of interest.

The work of Desaymard (72) showed that specific IgE tolerance could be developed to the DNP hapten by treatment with highly substituted DNP-LE (dinitrophenylated levan). As well, the tolerance was found to affect only the anti-DNP B cells of the IgE class, and the T cells were not involved at all.

Conjugates of Ficoll (M.W. 400,000) and DNP have also been used to establish an unresponsive state to DNP (73). In this case, however, suppression could only be achieved in the primary response and there was no effect on the secondary response.

In addition to the use of natural source high molecular weight carriers, several systems have been established using synthetic polymers, such as PEG (65-66). Also, a synthetic copolymer of D-glutamic acid and D-lysine has been used to investigate the establishment of tolerance (32,74). Also, recently, work has been done by von Specht using poly(N-vinylpyrrolidone) (PVP) (75). In this work, it was found that the 4-hydroxy-3-iodo-5-nitrophenacetyl (NIP) hapten could be attached to PVP and the conjugate was capable of inducing a

hapten specific suppression of both the primary and the secondary responses.

More, recently, Diner and his coworkers have done work on a new nonimmunogenic high molecular weight carrier, carboxymethyl cellulose (CMC) (76). In this study, conjugates of DNP and CMC were prepared and used to establish a state of tolerance to DNP with respect to the IgE response. The tolerant state created was specific for the anti-DNP response, was long-lasting and could be transferred into X-irradiated syngeneic recipients. However, there was no evidence supporting the presence of a suppressor T cell, but rather all of their findings supported a B cell form of tolerance.

Many different carrier substances have been used to establish specific states of unresponsiveness, in the hopes of finding a compound suitable for the treatment of human allergy patients. This thesis presents the initial studies performed using polyvinyl alcohol (PVA), a water-soluble polymer, as a nonimmunogenic carrier molecule for the establishment of a specific state of unresponsiveness in the IgE response. Conjugates of both the DNP and BPO haptens with PVA were prepared and their effects on both normal and sensitized animals were investigated. In addition, some preliminary cell transfer work was completed in an attempt to identify the mechanism(s) of tolerance induction operating in this system.

The preliminary studies were performed in the DNP-PVA system, as DNP is an excellent hapten for examining the IgE antibody response in the mouse. Thus the tolerogenic properties of the carrier PVA molecule could be easily investigated,

through the examination of the effect of the DNP-PVA conjugates on the response of the animals to DNP. The DNP hapten is of no importance in the study of clinical allergies, however, and it was for this reason that the tolerogenicity of the BPO-PVA conjugates was also investigated. The allergenicity of the BPO-PVA compounds was also examined, as this affects the usefulness of the compound in the treatment of clinical allergies. The ability of the conjugates to provide protection against systemic anaphylaxis was also tested, since this also has implications for the therapeutic use of the compound.

Thus, the immediate goal of this study was to investigate the tolerogenic properties of DNP-PVA and BPO-PVA on the reaginic response of the mouse. Furthermore, the studies were designed to contribute to the general understanding of the reaginic response. As an extension of this work, however, it is hoped that eventually it will be possible to develop a suitable regime for the treatment of human allergy patients using PVA conjugates.

MATERIALS & METHODS

Animals

Inbred 6 to 10 week old (C57BL/6 X DBA/2)F_1 mice (designated as $B_6D_2F_1$ or simply BDF_1) were purchased from Jackson Laboratories, Bar Harbour, Maine. For passive cutaneous anaphylaxis (PCA) assays, random-bred hooded rats, supplied by Bio-Breeding Laboratories Ltd., Ottawa, were used.

Preparation of Amino Derivatives of PVA (PVA-NH_2)

Molecular weights of 10,000 and 14,000 daltons of the polyvinyl alcohol were used and these will be referred to as PVA_{10} and PVA_{14} respectively. These compounds were purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin. Ten grams of either PVA_{10} or PVA_{14} were dissolved in 200 ml of distilled water followed by the addition of 5 grams of cyanogen bromide (from Eastman Kodak, Rodchester, N.Y.). This activation reaction results in the release of H^+ ion and consequently, the pH must be monitored. The pH was maintained at 11.0 by the dropwise addition of 2N NaOH. Twenty minutes later, 10 grams of 1,6-hexane diamine (from Eastman Kodak, Rodchester, N.Y.) were added and the mixture was stirred at room temperature for 48 hours. This mixture was then exhaustively dialyzed against distilled water and then lyophilized.

Preparation of DNP Conjugates of PVA

A solution of 500 mg of PVA_{10}-NH_2 in 50 ml of 0.4M Na_2CO_3 (Fisher Chemical Company) was prepared and 500 mg of sodium

2,4-dinitrobenzene sulphonate was added (Baker Chemical Co., Phillipsburg, N.J.). The mixture was incubated at 37°C for 3 days with stirring. Some precipitates, that formed during the incubation, were removed with centrifugation at 18,000 rpm in a Sorvall RC2-B centrifuge for 20 minutes. The supernatant was passed through a gel filtration column to remove any unreacted DNP hapten (4 X 60 cm;Sephadex G-25). The eluant containing the conjugate was dialyzed against distilled water and lyophilized.

The precipitates collected during the centrifugation above were redissolved in distilled water, dialyzed and lyophilized. The degree of substitution of all DNP-PVA conjugates was determined by dissolving a small, known mass of the conjugate in 5 ml of distilled water and examining the solution spectrophotometrically at 360 nm using the molar absorption coefficient for DNP as 17,400. The degree of substitution of the soluble component was found to be 1.5 and of the precipitated component 2.4.

The conjugate $DNP_{3.1}$-PVA_{10} was prepared as above, except 0.2M Na_2CO_3 was used and the reaction mixture stirred at room temperature for 2 days. The conjugate $DNP_{4.3}$-PVA_{14} was prepared as for $DNP_{3.1}$-PVA_{10} but 750 mg of PVA_{14}-NH_2 was used in the reaction mixture.

Preparation of BPO Conjugates of PVA

$PVA\text{-}NH_2$ (400 mg) was dissolved in 20 ml of distilled water followed by the addition of 400 mg of penicillin G (potassium salt, Pfizer Company, Ltd., Montreal) and 4 ml of 1M K_2CO_3, added slowly. This reaction mixture was stirred for 2.5 hours at room temperature. Unreacted penicillin G was re-

moved by gel filtration on a Sephadex G-25 column (4 X 60 cm) and the sample was dialyzed against distilled water and then lyophilized. The degree of substitution of the PVA molecule with the BPO groups was determined by quantitative titration with mercuric chloride solution, according to the method of Parker et al. (77) and was found to be 2.8. The other BPO_x-PVA conjugates of varying degrees of substitution were prepared in a similar manner, varying appropriately the concentration of penicillin G and allowing the reaction to procede for different periods of time. The conjugates used in this study are: $BPO_{0.7}$-PVA_{10}, $BPO_{1.1}$-PVA_{10}, BPO_2-PVA_{10}, $BPO_{2.8}$-PVA_{10}, $BPO_{1.3}$-PVA_{14}, $BPO_{2.7}$-PVA_{14} and $BPO_{3.9}$-PVA_{14}.

Preparation of Hapten-Protein Conjugates

The DNP_3-OA and DNP_{18}-BγG conjugates were synthesized according to the method of Ishizaka (22). Fifty mg of dinitrobenzene sulphonic acid (DNBS) and 50 mg of sodium carbonate were dissolved in 1 ml of distilled water and then were added to 5 ml of a solution of 100 mg of OA. The reaction mixture was stirred at room temperature for 4 hours and the unreacted hapten was removed using gel filtration through a column of Sephadex G-25 (4 X 60 cm).

The DNP_{18}-BγG was prepared in a similar manner using 100 mg of DNBS and 100 mg of Na_2CO_3 in 1 ml of distilled water. This was added to a 5 ml solution of 100 mg of BγG and stirred at room temperature for 48 hours.

The 2,4-dinitrophenylated extract of Ascaris suum (DNP-Asc) which contained 6.5 X 10^{-8}M DNP per mg of Asc, was prepared

by reacting 24 mg of sodium salt of 2,4-dinitrobenzene sulphonate with 46 mg of Asc in the presence of 46 mg of Na_2CO_3 in total volume of 5.5 ml of distilled water at 37°C for 2 hours.

The BPO-ovalbumin (BPO_4-OA), BPO-mouse gamma globulin (BPO_9-MγG) and BPO-bovine gamma globulin (BPO_{17}-BγG) conjugates were prepared as described in Lee and Sehon (63). The BPO_4-OA was prepared by reacting 250 mg of penicillin G (potassium salt) with 500 mg of ovalbumin in 25 ml of 0.5M K_2CO_3 at 37°C for 24 hours. The unreacted penicillin was removed from the conjugate by gel filtration through a column of Sephadex G-25 in PBS. The molar ratio of the benzylpenicilloyl groups to the ovalbumin was determined by quantitative titration with mercuric chloride as described by Parker et al. (77). The BPO_{17}-BγG conjugate used in the PCA challenges was prepared by reacting 250 mg of penicillin G with 500 mg of BγG under the same coupling conditions. The BPO_9-MγG was prepared by reacting, in the presence of 0.5M K_2CO_3, 50 mg of MγG with 25 mg of penicillin G in 6.6 ml at 4°C for 30 minutes. Unreacted penicillin G was removed as described above.

Immunization and Measurement of Reaginic and Hemagglutinating Antibody Responses

For optimal anti-DNP and anti-OA IgE responses, mice were intraperitoneally(i.p.) injected with the standard, low dose of 1 μg of DNP_3-OA suspended with 1 mg of freshly prepared aluminum hydroxide ($Al(OH)_3$) in 0.5 ml of phosphate buffered saline (PBS), as described by Levine and Vas (78). Hereafter, this dose, when administered by the i.p. route, will be referred

to as the sensitizing dose of DNP_3-OA. Mice were also sensitized i.p. with 10 µg of DNP-Asc in the presence of 1 mg of $Al(OH)_3$ in 0.5 ml of PBS for the induction of anti-DNP and anti-Asc antibodies of the IgE class. Similarly, mice were sensitized i.p. with the standard low dose of 10 µg of BPO_4-OA suspended with 1 mg of freshly prepared $Al(OH)_3$ in 0.5 ml of PBS. When administered by the i.p. route, this dose will hereafter be referred to as the sensitizing dose of BPO_4-OA.

For all of the above immunizations, four or five mice were treated identically and the sera of the mice from each group was pooled for the determination of the reaginic antibody titer. The concentration of hemagglutinating antibodies was also determined for the DNP system.

The average reaginic titers were determined by passive cutaneous anaphylaxis (PCA) assay performed in outbred hooded rats. In this assay, successive two-fold dilutions of the sera were made with saline. The backs of the rats were closely shaven and 50 µl of the various dilutions of sera were injected intradermally into the rats' backs, in duplicate. The animals were then challenged 24 hours later with an intravenous injection of 1 mg of DNP_{18}-BγG, OA, Asc, or BPO_{17}-BγG in 1 ml of saline containing 0.5 % Evans blue dye. The titer of the serum was then determined as the reciprocal of the highest dilution of the serum resulting in a reaction of 5 mm in diameter. All PCA results were reported as an average of two determinations.

To demonstrate the presence of antibodies other than those of the IgE class in the DNP system, the passive hemagglutination (HA) procedure was used. Conjugates of sheep red

blood cells (SBRC) with DNP_{18}-BγG or OA, synthesized by cross-linking with glutaraldehyde were utilized, as described by Avrameas (79). For this purpose, SRBC, which had been maintained in Alsever's solution, were washed three times, by gentle centrifugation, through PBS. To a solution of DNP_{18}-BγG or OA (40 mg/20 ml) 1 ml of packed cells was added. Then, 2.5 ml of 2.5 % solution of freshly prepared glutaraldehyde was added dropwise to the mixture with constant stirring. The reaction proceeded at room temperature for one hour and then the cells were washed 3 times with PBS. The volume of the cell suspension was then finally adjusted such that the concentration of cells was 10 times higher than that required for the HA test.

The assay was performed in micro hemagglutination plates using the Microtiter apparatus (Cooke Engineering Co., Alexandria, Virginia). Volumes of 25 µl of normal rabbit serum, which had been diluted 100-fold with PBS (i.e. 1 % NRS), were dispensed into each well of the plate with the aid of the microdiluter. Then, 25 µl of the serum to be tested was added and serially diluted in the plate. The suspension of SBRC (25 µl) coated with either DNP_{18}-BγG or OA, which had been diluted 14 times with 1 % NRS, was added to each well. The plates were then incubated at 37°C for 1 hour, after being shaken gently. The reciprocal of the highest dilution of antiserum at which agglutionation was just detectable became the hemagglutination titer.

The sensitivity and the specificity of the test was assured by the use of two separate controls on each plate. In the first control, normal mouse serum was serially diluted instead

of test serum to assure that hemagglutination was not occurring merely because of cross-linking with glutaraldehyde or some nonspecific factor. The sensitivity of the test was determined by the redetermination of the titer of a previously standardized rabbit or mouse serum containing anti-DNP or anti-OA antibodies.

Adoptive Cell Transfer

Spleen cells from sensitized or tolerized animals were transferred into X-irradiated (650 R) syngeneic recipients. Single cell suspensions of the spleen cells were prepared in Eagle's minimum essential medium (MEM) (Flow Laboratories Inc., Rockville, Md.), buffered with 20 mM hepes (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid, Calbiochem, San Diego, Calif.). These cell suspensions were then washed and transferred i.p. into the recipients which received a standard sensitizing dose of antigen in $Al(OH)_3$ within 4 hours of the cell transfer (61).

RESULTS

Following the preparation and spectrophotometric characterization of the various hapten-polyvinyl alcohol conjugates, several tests were designed to determine the effect of these compounds on the immunologic response of BDF_1 mice.

I. The DNP System

A. Effect of Dose of $DNP_{3.4}$-PVA_{10} on Anti-DNP IgE Response.

In this experiment, test groups of mice were given an i.p. injection of various quantities of $DNP_{3.4}$-PVA_{10}, 6 days prior to sensitization. Control mice did not receive anything at this time. The results are shown in Table I and clearly show that the anti-DNP IgE response is specifically suppressed, whereas the anti-OA IgE response is not affected. Also, the results indicate that some degree of suppression of the anti-DNP response occurs with a relatively low dose of the conjugate (200 μg), but more significant suppression is displayed with a dose of 1 mg of the conjugate.

B. Effect of Dose of $DNP_{4.3}$-PVA_{14} on Anti-DNP IgE Response.

A similar experiment was done to test the effect of dose of the conjugate $DNP_{4.3}$-PVA_{14}. The test mice were given an i.p. injection of varying amounts of tolerogen 7 days prior to the administration of the sensitizing dose of DNP_3-OA. Again, the results (found in Table II) indicate that while a low dose of the conjugate (250 μg) produces a slight suppression

TABLE I

EFFECT OF DOSE OF $DNP_{3.4}$-PVA_{10} ON SUPPRESSION OF ANTI-DNP IgE RESPONSES

DOSE OF TOLEROGEN*	PCA TITERS			
	PRIMARY		SECONDARY	
	ANTI-DNP	ANTI-OA	ANTI-DNP	ANTI-OA
0.20 mg	120	790	480	3410
0.50 mg	40	1480	270	3550
1.0 mg	10	440	90	3550
1.5 mg	20	1660	110	3630
2.0 mg	10	1780	80	3550
CONTROL MICE	810	940	1510	3310

*Test mice were injected i.p. with varying doses of the tolerogen 6 days prior to the sensitization with DNP_3-OA in the presence of $Al(OH)_3$. The primary IgE responses were determined on day 14 after sensitization. All mice were sensitized again on day 28 for the secondary response determination. The secondary anti-DNP and anti-OA responses were measured 7 days after immunization.

TABLE II

EFFECT OF DOSE OF $DNP_{4.3}$-PVA_{14} ON SUPPRESSION OF ANTI-DNP IgE RESPONSES

DOSE OF TOLEROGEN*	PCA TITERS			
	PRIMARY		SECONDARY	
	ANTI-DNP	ANTI-OA	ANTI-DNP	ANTI-OA
0.25 mg	90	830	330	3310
0.50 mg	80	1280	150	1450
0.75 mg	20	510	70	1120
1.0 mg	10	380	70	2460
1.5 mg	20	1410	40	3160
2.0 mg	< 10	160	10	2460
CONTROL MICE	380	500	870	1180

*Test mice were injected i.p. with varying doses of the tolerogen 7 days prior to sensitization with DNP_3-OA in the presence of $Al(OH)_3$. The primary IgE responses were determined on day 14 after sensitization. All mice were sensitized again on day 28 for the secondary responses. The secondary anti-DNP and anti-OA IgE responses were measured 7 days after immunization.

of the anti-DNP response, complete suppression is not achieved until a dose of about 1 mg is administered.

Because of these preliminary results, most future treatments of the mice with the conjugates to suppress the anti-DNP response consisted of a single dose of 1 mg injected via the i.p. route.

C. Suppression of the Induction and Abrogation of an Ongoing Anti-DNP IgE Response with $DNP_{3.4}$-PVA_{10}.

The next experiments that were performed were designed to afford a closer look at the effect of $DNP_{3.4}$-PVA_{10}. The effect of the tolerogen (the polyvinyl alcohol conjugates displaying a suppression of the anti-hapten response were referred to as tolerogens) on both the primary and secondary responses in both normal and primed animals was examined.

In the first experiment, normal mice were given a tolerizing dose of 1 mg of $DNP_{3.4}$-PVA_{10} 7 days prior to sensitization. Control mice received the sensitizing dose of DNP_3-OA only. PCA titers were determined several times following the immunization. The results can be seen in Figure 1. The graph clearly shows that the administration of the tolerogen on day -7 completely suppresses the primary anti-DNP response of the test mice. At the same time, however, the anti-OA response of these test animals is not affected by the tolerogen treatment. These results also indicate, to a certain extent, the long-lasting nature of the tolerogen treatment. Eight weeks after the initial injection of $DNP_{3.4}$-PVA_{10}, the test mice's anti-DNP response is still completely suppressed.

Fig.1. Suppression of the induction of anti-DNP IgE response

All mice received an i.p. sensitizing dose of 1 μg DNP_3-OA in presence of 1 mg of $Al(OH)_3$. Mice in test group received an additional i.p. injection of 1 mg $DNP_{3.4}$-PVA_{10} 7 days prior to sensitization. The IgE responses specfic for the DNP determinant (Δ---Δ) and for the carrier OA (o---o) are expressed in terms of PCA titers.

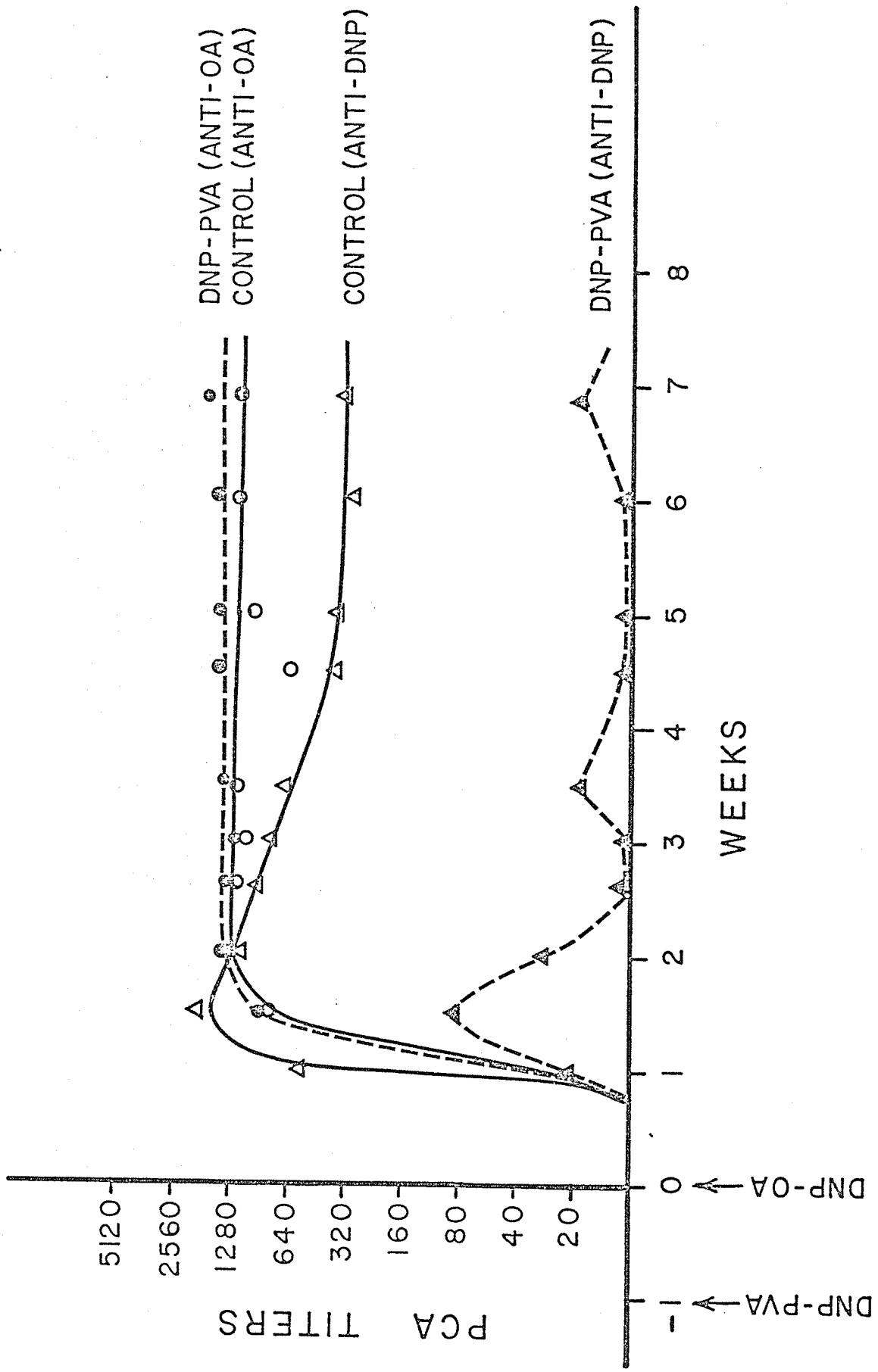

DNP-PVA (ANTI-OA)
CONTROL (ANTI-OA)
CONTROL (ANTI-DNP)
DNP-PVA (ANTI-DNP)
PCA TITERS
5120
2560
1280
640
320
160
80
40
20
WEEKS
DNP-OA
DNP-PVA

Figure 1.

Fig.2. Abrogation of an ongoing anti-DNP IgE response with $DNP_{3.4}$-PVA_{10}

All mice were sensitized with 1 µg DNP_3-OA in presence of 1 mg $Al(OH)_3$. Mice in the test group received an additional i.p. injection of 1 mg $DNP_{3.4}$-PVA_{10} 14 days after sensitization. The IgE responses specific for the DNP determinant and the carrier are represented by triangles and circles, respectively.

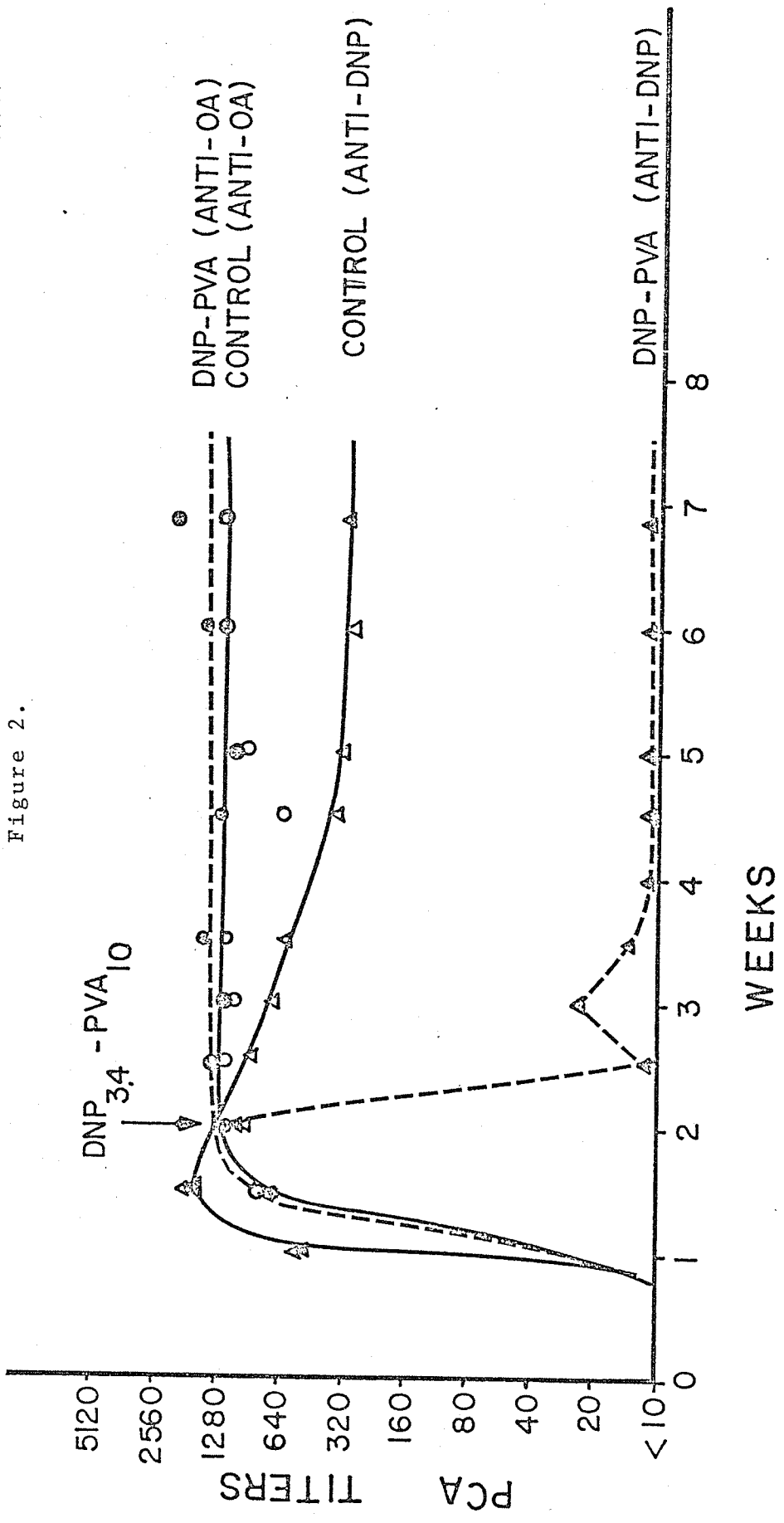

DNP-PVA (ANTI-OA)
CONTROL (ANTI-OA)
CONTROL (ANTI-DNP)
DNP-PVA (ANTI-DNP)
DNP3,4-PVA10
PCA TITERS
5120
2560
1280
640
320
160
80
40
20
<10
0
1
2
3
4
5
6
7
8
WEEKS

Figure 2.

Similarly, an experiment was performed to examine the effect of the tolerogen ($DNP_{3.4}$-PVA_{10}) on an ongoing anti-DNP reaginic response. In this situation, the tolerizing dose of 1 mg of $DNP_{3.4}$-PVA_{10} was administered i.p. 14 days after sensitization. Control mice received nothing at this time and the PCA titers were determined several times following the tolerization to determine the effect. Figure 2 illustrates the results and clearly shows the complete abrogation of the ongoing anti-DNP IgE response. Again the effect of the tolerogen is longlasting and as well, the anti-OA response of the test animals is not affected by the treatment with the tolerogen. The results from these two experiments are also tabulated in Tables III and IV.

D. Effect of Time of Injection of $DNP_{3.1}$-PVA_{10} on Reaginic Antibody Formation.

As a further extension of this work, two more experiments were designed to examine the effect of injecting the tolerogen at different times during the course of the reaginic response of the mouse. In one experiment, 1 mg of $DNP_{3.1}$-PVA_{10} was injected i.p. into mice at various times, from 14 days prior to sensitization to 24 days following sensitization. Both the primary anti-DNP and anti-OA responses (measured 14 days after sensitization) and the secondary responses (measured on day 7 after the second immunization) were determined by PCA. The results clearly show that suppression of the anti-DNP response can be accomplished by an i.p. injection of the tolerogen at most times during the course of the reaginic response, with perhaps some-

TABLE III

SUPPRESSION OF THE INDUCTION OF ANTI-DNP IgE RESPONSE WITH $DNP_{3.4}$-PVA_{10}

TIME OF PCA	PCA TITERS			
	CONTROL GROUP		TEST GROUP*	
	ANTI-DNP	ANTI-OA	ANTI-DNP	ANTI-OA
DAY 7	540	1660	20	10
DAY 10	1910	710	90	1660
DAY 14	870	980	30	1590
DAY 18	960	930	< 10	1320
DAY 21	690	870	< 10	920
DAY 24	680	1200	20	1380
DAY 31	330	580	< 10	1590
DAY 35	360	850	< 10	1550
DAY 42	280	1000	< 10	1700
DAY 48	360	1020	20	2040

*Test mice received an i.p. injection of 1 mg $DNP_{3.4}$-PVA_{10} 7 days prior to sensitization. All mice received the sensitizing dose of DNP_3-OA in the presence of $Al(OH)_3$ on day 0.

TABLE IV

ABROGATION OF AN ONGOING ANTI-DNP IgE RESPONSE WITH $DNP_{3.4}$-PVA_{10}

TIME OF PCA	PCA TITERS			
	CONTROL GROUP		TEST GROUP*	
	ANTI-DNP	ANTI-OA	ANTI-DNP	ANTI-OA
DAY 7	540	1660	540	1660
DAY 10	1910	710	1910	710
DAY 14	870	980	870	980
DAY 18	960	930	< 10	1280
DAY 21	690	870	30	930
DAY 24	680	1200	10	1590
DAY 31	330	580	< 10	1280
DAY 35	360	850	< 10	810
DAY 42	280	1000	< 10	1550
DAY 48	360	1020	< 10	3240

*All mice received the sensitizing dose of DNP_3-OA in the presence of $Al(OH)_3$ on day 0. The test group received an additional i.p. injection of 1 mg $DNP_{3.4}$-PVA_{10} on day 14, following the PCA determination on day 14.

TABLE V

EFFECT OF TIME OF INJECTION OF $DNP_{3.1}$-PVA_{10} ON REAGINIC ANTIBODY FORMATION

TIME OF TREATMENT*	PCA TITERS			
	PRIMARY		SECONDARY	
	ANTI-DNP	ANTI-OA	ANTI-DNP	ANTI-OA
DAY -14	100	1740	110	6030
DAY -10	100	1100	50	6030
DAY -7	100	1580	60	4470
DAY -3	40	950	60	2290
DAY -1	50	400	60	2140
DAY 0	80	670	90	2140
DAY 1	360	1100	400	3800
DAY 4	190	1050	490	3700
DAY 7	160	1000	640	4680
DAY 14	(580	2340)**	80	3470
DAY 24	(580	2340)**	90	3630
CONTROL MICE	580	2340	1100	3980

*Animals were treated with the tolerogen (1 mg $DNP_{3.1}$-PVA_{10}, i.p.) on the same day (i.e.day 0), or before or after immunization with DNP_3-OA in the presence of $Al(OH)_3$. The primary IgE responses were determined on day 14 after immunization. For induction of secondary responses, all mice were sensitized with DNP_3-OA in the presence of $Al(OH)_3$ on day 28 and the PCA titers were determined 7 days later.

**These PCA titers represent the primary IgE responses prior to the injection of the tolerogen.

what better suppression displayed with tolerization on days -3 to 0 (Table V).

E. Effect of Time of Injection of $DNP_{4.3}$-PVA_{14} on Reaginic Antibody Formation.

The comparable experiment was also performed using $DNP_{4.3}$-PVA_{14} as the tolerogen. Again, the various groups of test mice received 1 mg of $DNP_{4.3}$-PVA_{14} i.p. at different times during the course of the reaginic response. Again, both the primary and secondary anti-DNP and anti-OA responses were determined using PCA. The results are tabulated in Table VI. As can be seen from the Table, the results indicate that tolerization can be performed at most times during the course of the response and produce a significant suppression of the anti-DNP response. At the same time, in both of these experiments, the anti-OA response was unaffected by the treatment with the tolerogen. The tolerogen is thus able to very efficiently suppress a primary anti-DNP response and is similarly able to abrogate a well established anti-DNP response without interfering with the anti-OA response.

F. Specificity of Immunosuppression by $DNP_{2.4}$-PVA_{10}.

Having firmly esablished the ability of the DNP-PVA conjugates to suppress an anti-DNP response in mice, an experiment was designed to examine the specificity of the suppression of the anti-DNP response. Two experiments were designed and run simultaneously. In the first experiment, all mice were sensitized with DNP-Asc on day 0 and with DNP_3-OA on day 28. In addition, the control group received an i.p. injection of

TABLE VI

EFFECT OF TIME OF INJECTION OF $DNP_{4.3}$-PVA_{14} ON REAGINIC ANTIBODY FORMATION

TIME OF TREATMENT*	PCA TITERS			
	PRIMARY		SECONDARY	
	ANTI-DNP	ANTI-OA	ANTI-DNP	ANTI-OA
DAY -14	90	1580	70	3160
DAY -10	210	1580	220	3800
DAY -7	30	1280	150	2950
DAY -3	30	1320	30	2400
DAY -1	50	1280	50	2630
DAY 0	30	1280	20	4170
DAY 1	90	1510	80	6610
DAY 4	70	1380	90	3980
DAY 7	190	2340	330	5750
DAY 14	(1050	1320)**	70	5890
DAY 21	(1050	1320)**	180	6460
CONTROL MICE	1050	1320	1510	3090

*Animals were treated with the tolerogen (1 mg $DNP_{4.3}$-PVA_{14}, i.p.) on the same day (i.e.day 0), or before, or after i.p. immunization with DNP_3-OA in the presence of $Al(OH)_3$. The primary IgE responses were determined on day 14 after immunization. For the induction of secondary responses, all mice were sensitized with DNP_3-OA in the presence of $Al(OH)_3$ on day 28 and the PCA titers were determined 7 days later.

**These PCA titers represent the primary IgE responses prior to the injection of the tolerogen.

TABLE VII

SPECIFICITY OF IMMUNOSUPPRESSION BY $DNP_{2.4}$-PVA_{10}

	GROUP	TREATMENT* DAY	TREATMENT* COMPOUND	PCA TITERS DAY	ANTI-DNP	ANTI-OA	ANTI-ASC
EXPERIMENT 1	CONTROL	-2	SALINE				
		0	DNP-OA	7	< 10	< 10	N.T.**
				14	1700	1820	N.T.
		28	DNP-ASC	35	3470	3160	20
				42	2690	830	70
	TEST	-2	DNP-PVA				
		0	DNP-OA	7	< 10	< 10	N.T.
				14	20	1740	N.T.
		28	DNP-ASC	35	< 10	1700	10
				42	< 10	760	80
EXPERIMENT 2	CONTROL	-2	SALINE				
		0	DNP-ASC	7	< 10	N.T.	< 10
				14	300	N.T.	100
		28	DNP-OA	35	3540	80	370
				42	3500	480	320
	TEST	-2	DNP-PVA				
		0	DNP-ASC	7	< 10	N.T.	< 10
				14	< 10	N.T.	80
		28	DNP-OA	35	< 10	20	240
				42	20	810	80

*In experiment 1, all mice received sensitizing doses of DNP_3-OA and DNP-Asc in the presence of $Al(OH)_3$ on days 0 and 28 respectively. The test mice received additional injections of 1 mg of $DNP_{2.4}$-PVA_{10} on day -2. In experiment 2, all mice were immunized with sensitizing doses of DNP-Asc and DNP_3-OA in the presence of $Al(OH)_3$ on days 0 and 28 respectively. The test mice were pretreated with 1 mg of $DNP_{2.4}$-PVA_{10} on day -2.

**N.T. = not tested since the animals were not immunized with the corresponding carriers.

saline 2 days before the initial sensitization, while the test group received 1 mg of $DNP_{2.4}$-PVA_{10} i.p. at this time. In the analogous experiment, mice received a sensitizing dose of DNP_3-OA on day 0 and DNP-Asc on day 28. The same pretreatment of test and control mice occurred two days before the initial immunization.

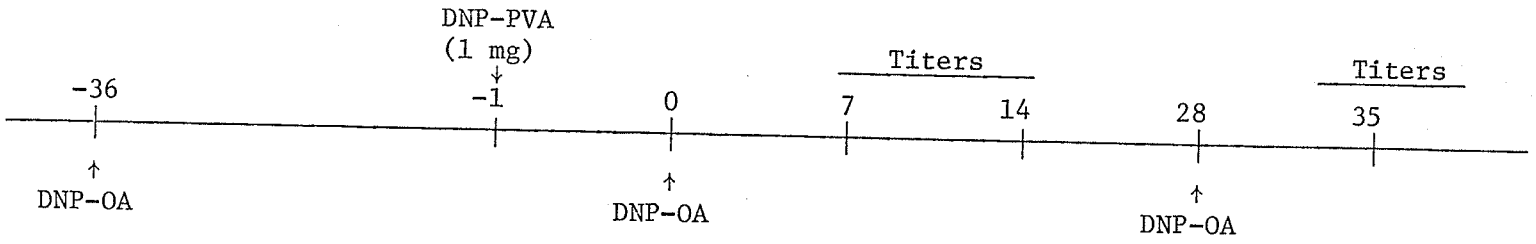
The results of these experiments are illustrated in Table VII. The results show that the suppression of the anti-DNP IgE response by the $DNP_{2.4}$-PVA_{10} conjugate is specific for the DNP hapten group and the response is suppressed irrespective of the carrier protein used in the immunization. At the same time, the reaginic response to both carrier proteins occurs unaffected by the presence of the tolerogen.

G. Abrogation of Hemagglutinating and Reaginic Antibody Responses with DNP-PVA in Sensitized Mice.

The suppression and abrogation of anti-DNP reaginic response is clearly accomplished by the administration of DNP-PVA conjugates. Since antibodies of the IgE class are not the only ones produced in response to the sensitizing dose of DNP_3-OA, the effect of the conjugates on the hemagglutinating antibodies was also examined. For this experiment, primed mice were used, having been given the sensitizing dose of DNP_3-OA 36 days previously. One day prior to resensitization, the first group received 1 mg of $DNP_{1.3}$-PVA_{10} i.p. At this time, the second test group received 1 mg of $DNP_{1.4}$-PVA_{14} i.p. and the control group received antigen only. Both the PCA and HA titers were determined 7 and 14 days later and 7 days after the third

TABLE VIII

ABROGATION OF HEMAGGLUTINATING AND REAGINIC ANTIBODY RESPONSES WITH DNP-PVA IN SENSITIZED MICE



GROUP	TREATMENT OF MICE SENSITIZED ON DAY -36*	DAY	HA TITERS ANTI-DNP	HA TITERS ANTI-OA	PCA TITERS ANTI-DNP	PCA TITERS ANTI-OA
CONTROL	Day 0: DNP-OA	7	32	128	1900	1700
		14	64	128	1700	3020
	Day 28: DNP-OA	35	128	256	1580	3020
TEST-I	Day -1: $DNP_{1.3}$-PVA_{10}					
	Day 0: DNP-OA	7	4	64	180	1740
		14	4	128	70	1660
	Day 28: DNP-OA		8	512	70	2480
TEST-II	Day -1: $DNP_{1.4}$-PVA_{14}					
	Day 0: DNP-OA	7	Nil	128	20	1990
		14	Nil	128	70	3310
	Day 28: DNP-OA	35	8	256	330	2560

*All mice were sensitized with the standard dose of DNP_3-OA on days -36, 0 and 28. Mice in test groups received additional i.p. injection of 1 mg $DNP_{1.3}$-PVA_{10} (TEST-I) or $DNP_{1.4}$-PVA_{14} (TEST-II) on day-2.

sensitization which occurred on day 28. The results of this experiment are shown in Table VIII. This Table definitely indicates that not only are these tolerogens capable of abrogating an existing reaginic anti-DNP response, but also are able to eliminate an ongoing hemagglutinating anti-DNP response. Also, once more, the anti-OA response, both hemagglutinating and reaginic, is not affected by the treatment with the tolerogen.

H. Tolerogenicity of DNP_x-PVA_{10} Conjugates with Different Epitope Densities in Sensitized Mice.

At this time, it was clear that the PVA conjugates of DNP were highly tolerogenic, but it had not been determined at what epitope density a conjugate became tolerogenic. Consequently, an experiment was designed to determine the effect of different epitope densities on the tolerogenicity of the DNP-PVA conjugates. Test mice were treated with either $DNP_{0.5}$-PVA_{10}, DNP_1-PVA_{10}, $DNP_{3.1}$-PVA_{10} or native PVA_{10}, while control mice received only PBS. Sensitized mice, which had received DNP_3-OA in alum 42 days earlier, were used and mice were resensitized 1 week and 4 weeks after having received 1 mg of free PVA_{10}, 1 mg of the DNP_x-PVA_{10} conjugate or 0.5 ml of PBS (controls). The secondary and tertiary anti-DNP and anti-OA IgE responses were then determined by PCA 7 days after each sensitization. The results can be seen in Table IX. Clearly, free PVA_{10} has no effect on either the anti-DNP or the anti-OA secondary and tertiary responses. The lightly haptenated conjugate ($DNP_{0.5}$-PVA_{10}) caused a moderate suppression of the anti-DNP secondary

TABLE IX

TOLEROGENICITY OF DNP_x-PVA_{10} CONJUGATES WITH DIFFERENT EPITOPE DENSITY IN SENSITIZED MICE

GROUP	COMPOUND INJECTED*	PCA TITERS**			
		SECONDARY		TERTIARY	
		ANTI-DNP	ANTI-OA	ANTI-DNP	ANTI-OA
I	PBS	3470	3470	5120	6160
II	PVA_{10}	3310	3470	5750	6610
III	$DNP_{0.5}$-PVA_{10}	830	3470	3390	5390
IV	DNP_1-PVA_{10}	< 10	3160	< 10	6610
V	$DNP_{3.1}$-PVA_{10}	< 10	3390	< 10	6160

*All mice were presensitized with DNP_3-OA 42 days prior to i.p. injections of either 0.5 ml PBS, 1 mg of free PVA_{10} or DNP_x-PVA_{10} conjugates of different epitope density. One and four weeks after the injection of DNP_x-PVA_{10}, all mice were sensitized with the standard doses of DNP_3-OA.

**The secondary and tertiary IgE responses were determined by PCA assay 7 days after sensitization with DNP_3-OA.

response and only weak suppression of the tertiary response. However, both the DNP_1-PVA_{10} and the $DNP_{3.1}$-PVA_{10} conjugates produced complete suppression of both the secondary and tertiary anti-DNP responses, with no effect on the anti-OA response. Thus it seems that an epitope density of at least one DNP group per molecule of PVA is necessary for the complete suppression of the anti-DNP IgE response.

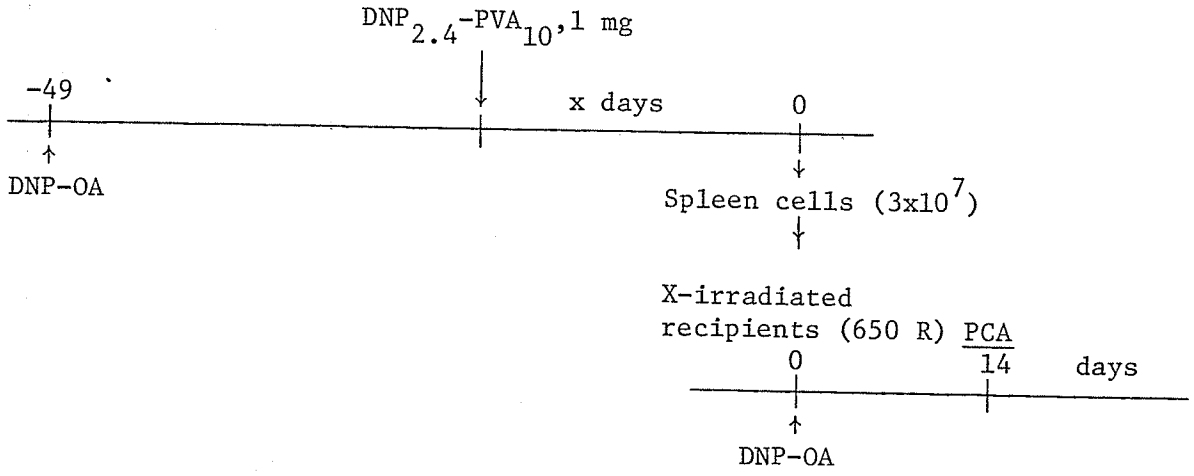
I. Maintenance of Unresponsiveness in Adoptive Cell Transfer.

Having established a system of unresponsiveness, which is easy to establish and maintain, it was decided to investigate the possibility of maintaining the unresponsiveness in cell transfers. Mice which had been sensitized 49 days prior to the cell transfer were used, and various test groups received in addition, 1 mg of $DNP_{2.4}$-PVA_{10} at different times before the transfer. The cells (3×10^7) were transferred into X-irradiated recipients (650 R) and the mice received a sensitizing dose of DNP_3-OA within 4 hours of the transfer. Anti-DNP and anti-OA IgE titers were determined 14 days later using PCA.

The results of this experiment are found in Table X. The cells from mice receiving the tolerizing dose of $DNP_{2.4}$-PVA_{10} 5 hours to 1 day prior to cell transfer, were unable to maintain the unresponsive state in the recipients. The cells from mice receiving this tolerizing dose 2 days and greater prior to the cell transfer were able to maintain the state of unresponsiveness in the recipients. Thus, it appears that 2 days are required for the establishment of tolerance in the

TABLE X

MAINTENANCE OF UNRESPONSIVENESS IN ADOPTIVE CELL TRANSFER SYSTEM



TIME OF TREATMENT PRIOR TO CELL TRANSFER *	PCA TITERS**	
	ANTI-DNP	ANTI-OA
5 hours	900	1910
1 day	920	1950
2 days	100	2000
3 days	300	1910
4 days	80	1950
7 days	80	1900
14 days	90	1800
CONTROL MICE	1430	2400

*Donors of spleen cells were preimmunized with a sensitizing dose of DNP_3-OA in the presence of $Al(OH)_3$ on day -49. A single dose of 1 mg of $DNP_{2.4}$-PVA_{10} was injected i.p. into these donors at various times prior to transfer of their spleen cells ($3x10^7$) intoX-irradiated (650 R) recipients. These recipients were challenged within 4 hours with a sensitizing dose of DNP-OA.

**The PCA titers were assayed 14 days after cell transfer.

Fig. 3. Active suppression of anti-DNP IgE response of primed cells with tolerized cells in adoptive transfer system

Different combinations of spleen cells from primed (S_p) and tolerized (S_t) mice, as indicated in the Fig., were transferred into X-irradiated (650 R) recipients. The primed donors were sensitized with DNP_3-OA 30 days befor cell transfer whereas the tolerized donors received a single injection of 1 mg $DNP_{2.4}$-PVA_{10} 7 days prior to cell transfer. The IgE responses to both the hapten and the carrier protein, represented by the hatched and open bars respectively, were measured in terms of PCA titers 14 days after cell transfer.

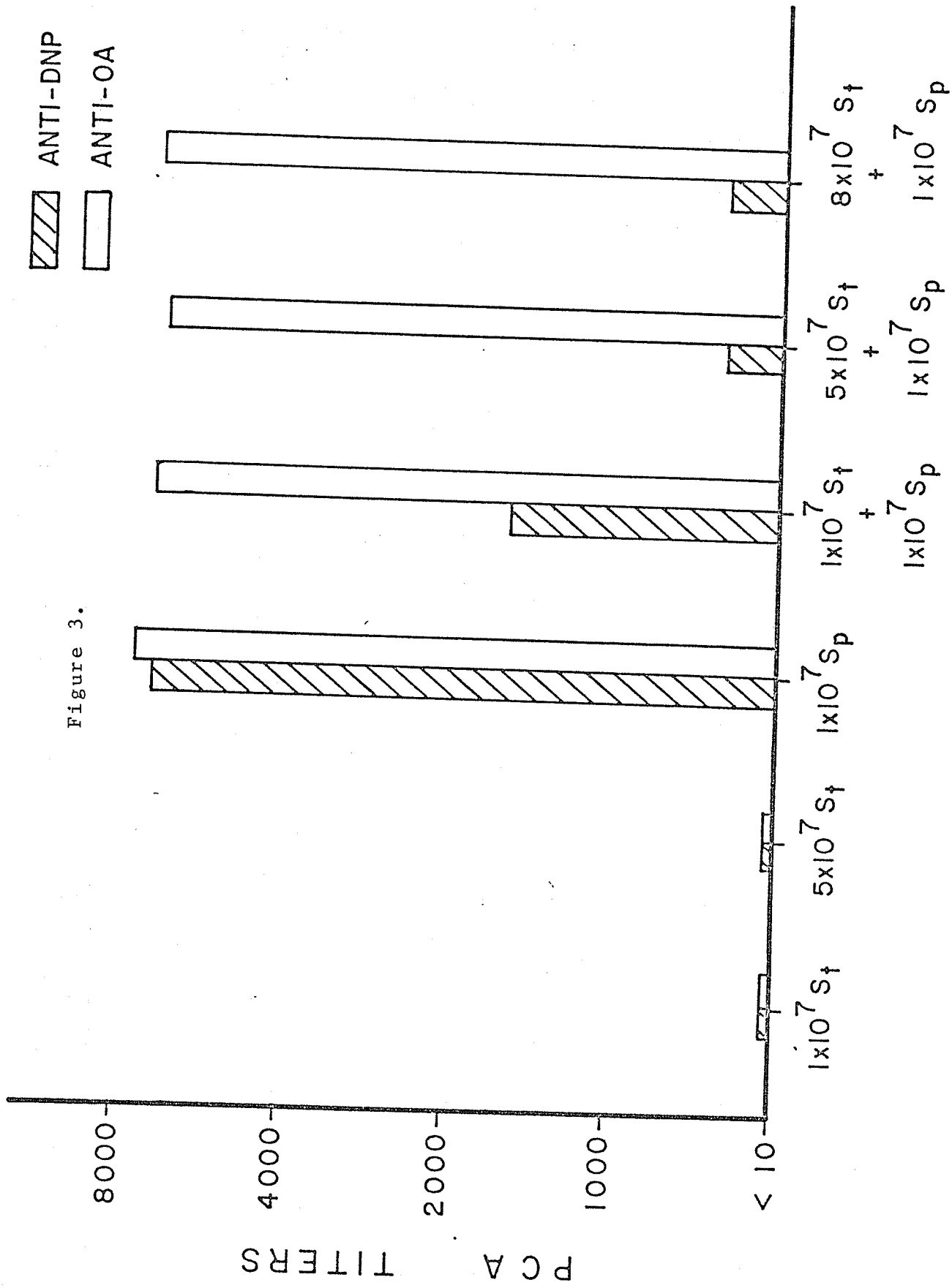

ANTI-DNP
ANTI-OA
PCA TITERS
8000
4000
2000
1000
< 10
1x10^7 S_t
5x10^7 S_t
1x10^7 S_p
1x10^7 S_t + 1x10^7 S_p
5x10^7 S_t + 1x10^7 S_p
8x10^7 S_t + 1x10^7 S_p
CELLS TRANSFERRED

Figure 3.

mice using DNP-PVA conjugates.

J. Acitve Suppression of Anti-DNP IgE Response of Primed Cells with Tolerized Cells in Adoptive Transfer System.

To investigate the possibility that suppressor cells were being produced in this system, an adoptive cell transfer system was used. Different combinations of spleen cells from tolerized and primed animals were transferred into X-irradiated (650 R) recipients. Primed spleen cells were obtained from animals that had received the sensitizing dose of DNP_3-OA 30 days before cell transfer. Tolerized spleen cells were from mice that had received 1 mg of $DNP_{2.4}$-PVA_{10} 7 days prior to the cell transfer. The results are illustrated in Figure 3 and clearly show that the tolerized spleen cells remain unresponsive following cell transfer. Also, the results indicate suppression of the anti-DNP IgE response of the primed cells by the presence of the tolerized cells in the recipients. Thus, there appears to be a DNP-specific suppressor cell present in the cell population following treatment with the $DNP_{2.4}$-PVA_{10} conjugate. The suppressor cell appears to be DNP-specific, as the anti-OA response is again unaffected.

K. Prevention of Systemic Anaphylaxis with $DNP_{2.4}$-PVA_{10} in Sensitized Mice.

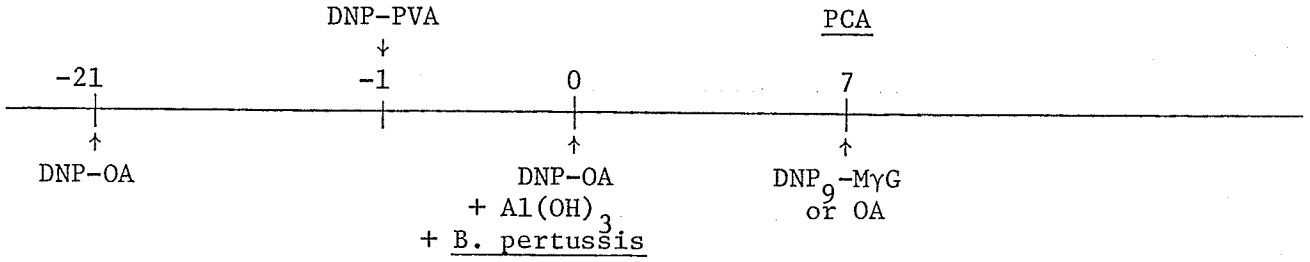
These conjugates are obviously able to suppress the initiation of an anti-DNP IgE response and are also able to abrogate an ongoing anti-DNP response. The presence of IgE in an animal is capable of causing a systemic reaction, and it

*All mice received a sensitizing dose of DNP_3-OA + $Al(OH)_3$ 3 weeks (i.e. on day -21) before the experiment. Mice in the CONTROL group were resensitized on day 0 with 1 µg of DNP_3-OA suspended in 1 mg of $Al(OH)_3$ and 8×10^9 B. pertussis. Mice in the TEST group received an additional i.p. injection of 1 mg of $DNP_{2.4}$-PVA_{10} one day before resensitization. After 7 days, mice were bled and challenged i.v. with 2 mg of DNP_9-MγG or OA, as indicated in the Table.

**The PCA titers refer to the reaginic antibodies in sera of mice 7 days after immunization.

TABLE XI

PREVENTION OF SYSTEMIC ANAPHYLAXIS WITH $DNP_{2.4}$-PVA_{10} IN SENSITIZED MICE



GROUP	TREATMENT OF MICE SENSITIZED ON DAY -21*	PCA TITERS**		SYSTEMIC REACTION ON CHALLENGE WITH	
		ANTI-DNP	ANTI-OA	DNP_9-MγG	OA
CONTROL (10 mice)	Day 0: DNP-OA + $Al(OH)_3$ + B. pertussis Day 7: Challenge i.v. with 2 mg DNP-MγG or OA	3320	3470	Death in 41, 44, 44, 47 and 49 min.	Death in 11, 11, 15, 18 and 25 min.
TEST (10 mice)	Day -1: 1 mg $DNP_{2.4}$-PVA_{10} Day 0: DNP-OA + $Al(OH)_3$ + B. pertussis Day 7: Challenge i.v. with 2 mg DNP-MγG or OA	<10	2560	No symptoms (5 mice)	Death in 15, 18, 19, 19 and 21 min.

*Please see next page for legend.

is of great interest to determine if the immunosuppressed animals would be protected from this systemic reaction, when challenged i.v. with a multivalent DNP-protein conjugate (i.e. DNP_9-MγG) or OA. The inclusion of Bordetella pertussis (8×10^9 cells) in the antigen preparation, caused the sensitized mice to become susceptible to systemic anaphylaxis, which was lethal. Sensitized mice, (immunized 21 days earlier) were used and the test mice received 1 mg of $DNP_{2.4}$-PVA_{10} one day prior to the secondary immunization with DNP_3-OA in $Al(OH)_3$ and B. pertussis. After 7 days, anti-DNP and anti-OA IgE responses were determined by PCA and the mice were challenged i.v. with 2 mg of DNP_9-MγG or OA. The results are listed in Table XI and clearly show that the mice which received 1 mg of $DNP_{2.4}$-PVA_{10} were protected from systemic anaphylaxis when challenged with DNP_9-MγG, but not when challenged with OA. The immunosuppression induced by the $DNP_{2.4}$-PVA_{10} compound is thus highly specific.

II. The BPO System

The work performed using the DNP system, clearly indicated that PVA was a suitable carrier molecule for the induction of tolerance to small hapten groups. The dinitrophenyl group is an ideal prototype for haptens, but is of no clinical value for the application to human allergy problems. Consequently, tests were performed to determine if the PVA molecule could also be used to establish a state of unresponsiveness to the BPO hapten, the major determinant in human penicillin allergies.

Preliminary tests were designed to examine the general tolerogenic properties of the BPO-PVA conjugates, as was done in the DNP-PVA system. However, because the possibility exists that the BPO-PVA conjugates could be used in the treatment of human penicillin allergy patients, other properties of the conjugates also had to be examined. Human allergy patients are much more susceptible to systemic anaphylaxis, often resulting in death, than are mice displaying a reaginic antibody response. Thus, it was necessary to investigate the ability of the BPO-PVA conjugates to elicit PCA, to neutralize pre-existing anti-BPO IgE antibodies and to prevent systemic anaphylaxis. This was imperative, as these properties profoundly influence the suitability of the conjugates for the treatment of human allergy patients. The particular experiments studying these aspects of the tolerogenicity of the BPO-PVA conjugates are presented in sections D, E and F.

A. Suppression of the Induction of Anti-BPO IgE Response with BPO_2-PVA_{10} or $BPO_{2.7}$-PVA_{14}.

The tolerogenic compounds were prepared as described in the Materials and Methods and preliminary tests were designed to examine the effect of the BPO-PVA conjugates on the induction of a primary anti-BPO IgE response. Two conjugates, BPO_2-PVA_{10} and $BPO_{2.7}$-PVA_{14} were tested in two doses, 0.5 mg and 1 mg for each conjugate. The test groups received the conjugate i.p. 7 days prior to the sensitization at which time all mice received 10 µg of BPO_4-OA in 1 mg of $Al(OH)_3$ i.p. All mice received a secondary and a tertiary immunization on day 28 and day 56 respectively and PCA titers were determined 14 days after the primary immunization and 7 days after both the secondary and tertiary immunizations.

The results are tabulated in Table XII and clearly show that both doses of both conjugates suppress the primary and secondary anti-BPO IgE responses, without influencing the anti-OA IgE responses. Even after tertiary immunization, all test groups, except for the test group receiving 0.5 mg of BPO_2-PVA_{10}, are still greatly suppressed.

B. Abrogation of Anti-BPO IgE Response with BPO_2-PVA_{10} and $BPO_{2.7}$-PVA_{14} in Sensitized Animals.

The effect of the conjugates in sensitized animals is perhaps more important than in naive animals. Consequently, two parallel experiments were designed to study the effect of dose of BPO_2-PVA_{10} or $BPO_{2.7}$-PVA_{14}, on the abrogation of an ongoing anti-BPO IgE response. For this test, sensitized mice

TABLE XII

SUPPRESSION OF THE INDUCTION OF ANTI-BPO IgE RESPONSE WITH BPO_2-PVA_{10} OR $BPO_{2.7}$-PVA_{14}

TOLEROGEN INJECTED *	PCA TITERS					
	PRIMARY		SECONDARY		TERTIARY	
	ANTI-BPO	ANTI-OA	ANTI-BPO	ANTI-OA	ANTI-BPO	ANTI-OA
BPO_2-PVA_{10}, 0.5 mg	< 10	1450	20	3160	1260	5370
BPO_2-PVA_{10}, 1.0 mg	< 10	1580	< 10	2820	360	4360
$BPO_{2.7}$-PVA_{14}, 0.5 mg	< 10	1740	10	3980	360	6030
$BPO_{2.7}$-PVA_{14}, 1.0 mg	< 10	680	< 10	3020	320	5620
CONTROL MICE	30	1910	810	3980	2750	6920

*Mice were injected i.p. with BPO_2-PVA_{10} or $BPO_{2.7}$-PVA_{14} 7 days prior to sensitization with BPO_4-OA in the presence of $Al(OH)_3$. The primary IgE responses were determined on day 14 after sensitization. For the induction of secondary and tertiary IgE responses, all mice were immunized with sensitizing doses of BPO_4-OA on day 28 and 56, respectively, and the PCA titers were determined 7 days after sensitization.

TABLE XIII

ABROGATION OF ANTI-BPO IgE RESPONSE WITH BPO_2-PVA_{10} IN SENSITIZED ANIMALS

GROUP	TREATMENT*		PCA TITERS		
	DAY	COMPOUND	DAY	ANTI-BPO	ANTI-OA
CONTROL	0	BPO-OA	14	90	3100
	28	BPO-OA	35	870	3630
	56	BPO-OA	63	1450	4170
TEST 1	0	BPO-OA	14	90	3100
	21	BPO-PVA, 0.5 mg			
	28	BPO-OA	35	<10	5370
	56	BPO-OA	63	930	6920
TEST 2	0	BPO-OA	14	90	3100
	21	BPO-PVA, 0.75 mg			
	28	BPO-OA	35	<10	3240
	56	BPO-OA	63	<10	5370
TEST 3	0	BPO-OA	14	90	3100
	21	BPO-PVA, 1.0 mg			
	28	BPO-OA	35	<10	4470
	56	BPO-OA	63	360	6610

*All mice received sensitizing doses of BPO-OA on days 0, 28 and 56. Mice in the test groups received additional injections of 0.5 mg (TEST 1), 0.75 mg (TSET 2) or 1.0 mg (TEST 3) of BPO_2-PVA_{10} on day 21.

TABLE XIV

EFFECT OF DOSE OF $BPO_{2.7}$-PVA_{14} ON THE ABROGATION OF ANTI-BPO RESPONSE IN SENSITIZED MICE

GROUP	TREATMENT*		PCA TITERS		
	DAY	COMPOUND	DAY	ANTI-BPO	ANTI-OA
CONTROL	0	BPO_4-OA	14	70	4000
	28	BPO_4-OA	35	1050	4570
	56	BPO_4-OA	63	1350	3630
TEST 1	0	BPO_4-OA	14	70	4000
	21	$BPO_{2.7}$-PVA_{14}, 0.1 mg			
	28	BPO_4-OA	35	20	5750
	56	BPO_4-OA	63	790	4470
TEST 2	0	BPO_4-OA	14	70	4000
	21	$BPO_{2.7}$-PVA_{14}, 0.25 mg			
	28	BPO_4-OA	35	< 10	3800
	56	BPO_4-OA	63	100	3380
TEST 3	0	BPO_4-OA	14	70	4000
	21	$BPO_{2.7}$-PVA_{14}, 0.5 mg			
	28	BPO_4-OA	35	< 10	5370
	56	BPO_4-OA	63	90	2950
TEST 4	0	BPO_4-OA	14	70	4000
	21	$BPO_{2.7}$-PVA_{14}, 1.0 mg			
	28	BPO_4-OA	35	< 10	5750
	56	BPO_4-OA	63	100	2560

*All mice received sensitizing doses of BPO_4-OA on days 0, 28 and 56. Mice in the test groups received injections of various doses of $BPO_{2.7}$-PVA_{14} on day 21.

were used and on day 21, test mice received either 0.5 mg, 0.75 mg, or 1.0 mg of BPO_2-PVA_{10} or 0.1 mg, 0.25 mg, 0.5 mg or 1.0 mg of $BPO_{2.7}$-PVA_{14} i.p. All mice received a second sensitizing dose of BPO_4-OA 7 days later. A tertiary immunization was administered on day 56 and secondary and tertiary PCA titers were determined 7 days after these immunizations. The results are tabulated in Tables XIII and XIV.

The results in Table XIII definitely indicate the abrogation of an ongoing anti-BPO response with as little as 0.5 mg of BPO_2-PVA_{10}. The tertiary response is also suppressed, especially with the two higher doses, and the anti-OA IgE response is not affected at anytime. Similarly, the data in Table XIV indicates the $BPO_{2.7}$-PVA_{14}, even at the low dose of 0.1 mg is able to abrogate an ongoing anti-BPO IgE response with no effect on the anti-OA antibody response. A dose of at least 0.25 mg of $BPO_{2.7}$-PVA_{14} is required, however, if the abrogation of the anti-BPO IgE response is to be longlasting.

C. Tolerogenicity of BPO_x-PVA Conjugates with Different Epitope Densities in Sensitized Animals.

In establishing a state of tolerance in animals, not only is the dose of the tolerogen used important, but also the epitope density of the hapten on the carrier. In this study, mice were sensitized with 10 µg of BPO_4-OA in alum and then 21 days later, the test mice received an i.p. injection of 1 mg of BPO_x-PVA. Seven days later, all mice received a second sensitizing dose of BPO_4-OA. Epitope densities tested ranged from 0.7 to 2.8 for PVA_{10} and as well, two PVA_{14} conjugates were

TABLE XV

TOLEROGENICITY OF BPO_x-PVA CONJUGATES WITH DIFFERENT EPITOPE DENSITY IN SENSITIZED MICE

TREATMENT*	PCA TITERS	
	ANTI-BPO	ANTI-OA
$BPO_{0.7}$-PVA_{10}	280	6170
$BPO_{1.1}$-PVA_{10}	< 10	3800
$BPO_{2.0}$-PVA_{10}	< 10	3550
$BPO_{2.8}$-PVA_{10}	< 10	5750
$BPO_{2.7}$-PVA_{14}	< 10	3980
$BPO_{3.9}$-PVA_{14}	< 10	5120
CONTROL MICE	1050	4570

*All mice were presensitized with BPO_4-OA 21 days prior to i.p. injections of 1 mg of BPO_x-PVA_{10} or BPO_x-PVA_{14} conjugates of different epitope density. One week after the injection of BPO_x-PVA, all mice were sensitized with BPO_4-OA in the presence of $Al(OH)_3$ and PCA titers were determined 7 days later.

tested. The results are illustrated in Table XV. As can be seen in this Table, all epitope densities tested suppressed the response of the test mice and complete suppression was observed with epitope densities greater than 1.0. In all cases, the anti-OA IgE responses was unaffected by the treatment with BPO_x-PVA.

D. PCA Eliciting Efficiency of BPO_x-PVA Conjugates of Different Epitope Densities.

In this study, the ability of BPO_x-PVA compounds to elicit PCA reactions was examined. For this purpose, 0.1 ml of a standard mouse reaginic serum, which had been produced by i.p. immunization of mice with the sensitizing dose of BPO_4-OA, was two-fold serially diluted with PBS. This serum had a known anti-BPO titer of 760 and 50 µl of the diluted solutions were injected intradermally into the shaven backs of outbred hooded rats. Twenty-four hours later, the rats were i.v. challenged with a 1 ml solution containing 1 mg of a BPO_x-PVA conjugate and 0.5 % Evans blue dye. The rats were killed 30 minutes later and the PCA reactions determined.

The results of this test are tabulated in Table XVI and clearly show that di- and multivalent BPO-PVA conjugates elicit substantial PCA reactions in comparison to the control. The conjugates with low hapten densities (i.e. $BPO_{0.7}$-PVA_{10}, $BPO_{1.1}$-PVA_{10} and $BPO_{1.3}$-PVA_{14}) however, elicit very weak PCA reactions in comparison to that elicited by the multivalent BPO_9-MγG in the control.

E. Ability of BPO_x-PVA Conjugates to Neutralize Anti-BPO IgE Antibodies.

TABLE XVI

PCA ELICITING EFFICIENCY OF BPO_x-PVA CONJUGATES OF DIFFERENT EPITOPE DENSITY

CHALLENGING COMPOUND (1mg)*	ANTI-BPO PCA TITERS
$BPO_{0.7}$-PVA_{10}	60
$BPO_{1.1}$-PVA_{10}	80
$BPO_{2.0}$-PVA_{10}	230
$BPO_{2.8}$-PVA_{10}	200
$BPO_{1.3}$-PVA_{14}	80
$BPO_{2.7}$-PVA_{14}	260
BPO_9-MγG	760

*Random-bred hooded rats were sensitized i.d. with a mouse anti-BPO reaginic serum and were challenged 24 hours later by i.v. injection of 1 mg of the compounds shown in the Table together with Evans blue dye in PBS.

As an extension of the work done in the previous section, and experiment was devised to examine the ability of the various BPO-PVA conjugates to neutralize anti-BPO IgE antibodies *in vitro*. Volumes of 0.1 ml of a standard mouse reaginic serum, having an anti-BPO PCA titer of 640, were mixed *in vitro* with 0.1 ml of PBS containing various amounts of the BPO-PVA conjugates. The mixtures were then diluted to 1 ml with PBS and two-fold serial dilutions with PBS were prepared. Next, 0.1 ml of the various dilutions of the mixtures were injected intradermally into the shaven backs of outbred hooded rats for the PCA determinations. The rats were challenged i.v. 24 hours later with 1.0 ml of a solution containing 1 mg of BPO_9-MγG and 0.5 % Evans blue dye. The percentage of neutralization of reaginic antibodies by a given BPO-PVA conjugate, which had been added to the reaginic serum prior to sensitization of the rats, was calculated by comparing the PCA titer of the residual free murine reaginic antibodies (i.e. the titer determined for mixtures of reaginic antiserum containing the BPO-PVA conjugate) to the PCA titer of the reaginic serum in absence of BPO-PVA conjugates.

The results of this test are in Table XVII and clearly show that the conjugates with low epitope density (i.e. $BPO_{0.7}$-PVA_{10} and $BPO_{1.3}$-PVA_{14}) did not neutralize any PCA reactions at the concentrations tested. $BPO_{1.1}$-PVA_{10} neutralized only at high concentrations (100 µg) and BPO_2-PVA_{10} and $BPO_{2.7}$-PVA_{14} neutralized at 10 µg whereas $BPO_{2.8}$-PVA_{10} neutralized at 1 µg as a multivalent BPO-protein conjugate (i.e. similar to BPO_9-MγG).

TABLE XVII

THE ABILITY OF BPO_x-PVA CONJUGATES TO NEUTRALIZE ANTI-BPO IgE ANTIBODIES

COMPOUND ADDED TO REAGINIC SERUM *	% NEUTRALIZATION OF PCA AT		
	1 µg	10 µg	100 µg
$BPO_{0.7}$-PVA_{10}	0	0	0
$BPO_{1.1}$-PVA_{10}	0	0	70
$BPO_{2.0}$-PVA_{10}	0	70	100
$BPO_{2.8}$-PVA_{10}	70	100	100
$BPO_{1.3}$-PVA_{14}	0	0	0
$BPO_{2.7}$-PVA_{14}	0	70	70
BPO_9-MγG	50	100	100

*Various amounts of the compounds as listed, were added to 0.1 ml volumes of the mouse reaginic serum of known anti-BPO titer (PCA equals 640) and diluted to a volume of 1.0 ml. The residual free anti-BPO IgE antibodies were determined by PCA as described earlier in Materials and Methods.

F. Prevention of Systemic Anaphylaxis with $BPO_{1.6}$-PVA_{10} in Sensitized Animals.

From the previous experiments, it has been shown that an ongoing reaginic response can be abrogated by treating the sensitized animals with a BPO-PVA conjugate. The present study was designed to examine the possibility of protecting sensitized animals from systemic anaphylaxis upon challenge with a multivalent BPO-protein conjugate (BPO_9-MγG) or OA, by pretreatment with a BPO-PVA conjugate.

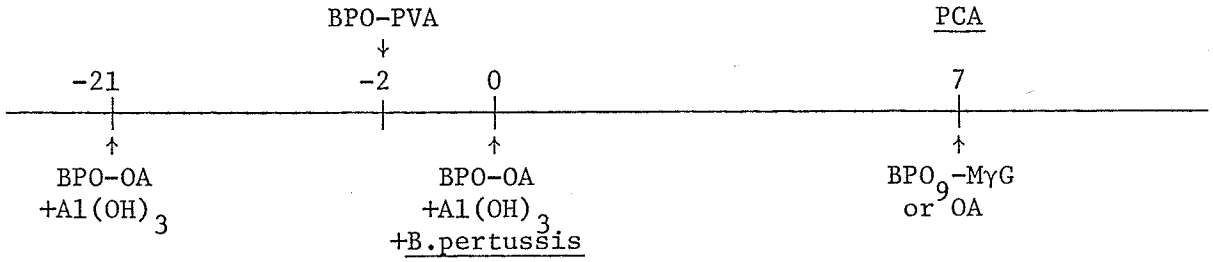
In order to demonstrate systemic anaphylaxis, B.pertussis must be used in the sensitizing antigen mixture in the immunization of the animals. All of the mice were presensitized with BPO_4-OA in $Al(OH)_3$ 21 days earlier and the test mice received 1 mg of $BPO_{1.6}$-PVA_{10} i.p. 2 days prior to the secondary sensitization, which included the B. pertussis. The results from this study are illustrated in Table XVIII and definitely show that the tolerization treatment completely suppressed the anti-BPO IgE response, whereas the anti-OA IgE antibody production was not affected. As well, and more importantly, the tolerized mice were protected from systemic anaphylaxis when challenged i.v. with BPO_9-MγG, whereas the control mice died within 22 minutes. This protection is highly antigen specific, however, and both groups died when challenged i.v. with OA.

*All mice were primed with a sensitizing dose of BPO_4-OA + $Al(OH)_3$ 21 days before the experiment. Mice in the CONTROL group were resensitized on day 0 with 10 µg of BPO_4-OA suspended in 1 mg of $Al(OH)_3$ and 8 X 10^9 B. pertussis. Mice in the TEST group received an additional i.p. injection of 1 mg of $BPO_{1.6}$-PVA_{10} one day before resensitization. After 7 days, mice were bled and challenged i.v. with 2 mg of BPO_9-MγG or OA, as indicated in the Table.

**The PCA titers refer to the reaginic antibodies in sera of mice 7 days after immunization.

TABLE XVIII

PREVENTION OF SYSTEMIC ANAPHYLAXIS WITH $BPO_{1.6}$-PVA_{10} IN SENSITIZED MICE



GROUP	TREATMENT OF MICE SENSITIZED ON DAY -21*	PCA TITERS**		SYSTEMIC REACTION ON CHALLENGE WITH	
		ANTI-BPO	ANTI-OA	BPO_9-MγG	OA
CONTROL (10 mice)	Day 0: BPO-OA + $Al(OH)_3$ + B. pertussis Day 7: Challenge i.v. with 2 mg BPO_9-MγG or OA	800	1780	Death in 14, 16, 18, 19 and 22 min.	Death in 10, 10, 11, 15 and 18 min.
TEST (10 mice)	Day -2: 1 mg $BPO_{1.6}$-PVA_{10} Day 0: BPO-OA + $Al(OH)_3$ + B. pertussis Day 7: Challenge i.v. with 2 mg BPO_9-MγG or OA	< 10	1900	No symptoms (5 mice)	Death in 10,11, 12, ,2 and 15 min.

*Please see next page for legend.

DISCUSSION

The work presented in this thesis clearly demonstrates that the use of polyvinyl alcohol as a nonimmunogenic carrier molecule could prove very valuable in the treatment of clinical allergies. The system developed using PVA as a carrier is only in its infancy at the moment, but already definite advantages to its use have been found. The PVA molecule and its conjugates are water-soluble which makes the preparation of conjugates and their subsequent administration extremely facile. Secondly, the tolerogenic effects of the conjugate are immunospecific and longlasting. Also, it appears that some of the conjugates that are capable of suppressing or abrogating a reaginic response in an animal are also prophylactic against systemic anaphylactic shock. Thus, it seems that the system has very definite potential.

In the past, considerable work has been done using nonimmunogenic or weakly immunogenic molecules as carriers; such as isologous γ-globulins (60,63), a copolymer of D-glutamic acid and D-lysine (34,74), Ficoll (73), levan (71-72), polyvinyl pyrrolidone (75), mycobacterium (67-69) and carboxymethyl cellulose (76). This work is based on the documentation that the release of pharmacologically active compounds from mast cells or basophils is the result of a triggering mechanism initiated by the cross-linking of IgE molecules (attached to the surface of these cells) by multivalent antigens (80-82). Consequently, if the elimination of the circulating pool of IgE molecules was possible, it would seem likely that the triggering of the mast

cells and basophils would also be eliminated. Using conjugates of various haptens and the above carriers, it has been possible to abrogate reaginic responses to the specific haptens. Similarly, in this work, the tolerogenic and water-soluble carrier, polyvinyl alcohol, has been used to study the response to both the DNP and BPO haptens.

It has been demonstrated in this work that pretreatment of mice with a single dose of 1 mg of DNP-PVA is sufficient to suppress the primary anti-DNP reaginic response (Tables I & II). As well, a primary anti-BPO reaginic response can be effectively suppressed by pretreatment with 1 mg of BPO-PVA (Table XII). More importantly, however, the conjugates of PVA with both DNP and BPO are capcable of abolishing a pre-existing anti-DNP or anti-BPO reaginic response (Tables I, II & XIII). Relatively low doses of the tolerogens were sufficient to completely abrogate an ongoing IgE response. A dose of 1 mg of conjugate was required for the complete elimination of an anti-DNP reaginic response, whereas only 0.1 mg of $BPO_{2.7}$-PVA_{14} completely abolished an ongoing anti-BPO reaginic response (Table XIV). The anti-DNP hemagglutinating antibody response was also suppressed by this treatment (Table VIII) and in other work, not presented here, a similar effect on the anti-BPO hemagglutinating antibody response following treatment with BPO-PVA was also found.

The effect of the tolerogens on the animals was found to be very longlasting as well. Experimentally, it was found that a single treatment with 1 mg of $DNP_{3.4}$-PVA_{10} was sufficient to suppress a primary response for 7 weeks (Fig. 1 & Table III) without any subsequent treatment. Similarly, the secondary

anti-DNP response was efficiently abrogated for a period of more than 6 weeks following a single injection of 1 mg of $DNP_{3.1}$-PVA_{10} (Fig. 2 & Table IV).

The degree of substitution of the hapten groups (either DNP or BPO) on the PVA molecule also has a definite influence on the tolerogenicity of the compound. In both the systems, several conjugates of different epitope density were tested to determine the effect on the ease of induction of tolerance by the conjugate. In each case, definite, although incomplete, suppression of the anti-hapten response was found with epitope densities less than 1.0. Thus, $BPO_{0.7}$-PVA_{10} and $DNP_{0.5}$-PVA_{10} suppressed the appropriate antibody response, but not completely. It was necessary to administer a conjugate with an epitope density of at least 1.0 before complete suppression of the anti-hapten IgE response was realized (Tables IX & XV).

The tolerogenicity of the conjugate is one important aspect in the use of these conjugates in the treatment of allergy. The other aspect which is equally important, is the allergenicity of the conjugates. For the conjugate to be effective in the treatment of allergy, it must have no ability, or at least very limited ability to react with antibodies already formed to the native allergen. Thus, a conjugate used in treating a penicillin allergy should not react efficiently with pre-existing anti-BPO reaginic antibodies, otherwise a systemic reaction could be elicited during the treatment.

The ability of BPO_x-PVA conjugates to react with anti-BPO reaginic antibodies was tested in two separate ways. The PCA eliciting efficiency (Table XVI) and the anti-BPO IgE antibody

neutralizing ability (Table XVII) were both examined. The low epitope density conjugates were found to have very limited ability to elicit PCA. This is a direct result of the low number of hapten groups attached to any one molecule of PVA, limiting the conjugate's ability to cross-link IgE antibody molecules on the surface of effector cells. Since the release of histamine and other pharmacologically active chemicals from the effector cells is mediated by the cross-linking of the IgE molecules on the cell surface, PVA molecules must have at least two hapten groups attached to them before they can effectively cause a PCA reaction. Similarly, the low epitope density conjugates had limited IgE antibody neutralizing ability. The results of both studies indicate that the allergenicity of the low epitope density conjugates (i.e. $BPO_{1.1}$-PVA_{10} and $BPO_{1.3}$-PVA_{14}) is very low, making them suitable compounds for the treatment of allergy victims.

The suppression of reaginic and hemagglutinating antibodies, as a result of the treatment with hapten-PVA conjugates, is highly specific. At no time has the anti-carrier response been affected, even with very high doses of the conjugates. Also, the time of injection of the PVA conjugates has been found to be essentially noncritical. Suppression has been achieved with injection of the tolerogen either before (days -14 to -1), after (days 1 to 21) or at the time of sensitization (day 0) for the DNP system (Tables V & VI). These Tables indicate that somewhat better suppression of the anti-DNP IgE response was achieved when the tolerogen was administered on days -3 to 0. However, this difference is not highly

significant (only a 2-3 fold difference in PCA titers between mice tolerized on either day -14 or day -3 with $DNP_{3.1}$-PVA_{10}, Table V) and it was generally found that suppression could be achieved with injection of the tolerogen at most times during the course of the reaginic response. Similarly, in the BPO system, tolerance was induced in both naive and sensitized animals (Tables XII & XIII). This longlasting, highly specific suppression was also evidenced by the fact that the suppression of the anti-DNP response could not be broken by sensitization with a different carrier molecule bearing the DNP hapten (Table VII).

In most of the other systems using a weakly immunogenic or nonimmunogenic carrier to establish a state of tolerance, the site of action of the suppression has been found to be the hapten specific B cell population. Thus, the use of either isologous γ-globulins or D-GL results in a form of B cell tolerance mediated by either receptor blockade (63,83) or central inactivation (74) mechanisms. Similarly, Diner et al. (76) induced a form of B cell tolerance using carboxymethyl cellulose as the carrier molecule.

In the DNP-PVA system, cell transfers have been performed in which spleen cells from tolerized animals were transferred into X-irradiated recipients. When the cells transferred originated from animals receiving the tolerization treatment 2 or more days prior to cell transfer, the suppression of the anti-DNP IgE response was transferred into the recipients (Table X). This result could support a form of B cell tolerance, but would also support a form of T cell tolerance. More

studies must be conducted in this area in order to establish the mechanism of tolerance induction that is operating in this system.

In the DNP-PVA system, it has been found that an admixture of spleen cells from primed and tolerized mice transferred into X-irradiated syngeneic recipients did not display a normal anti-DNP response upon sensitization. Thus, it is concluded that the treatment with DNP-PVA resulted in the generation of hapten specific active suppressor cells (Fig. 3.). These cells are currently under investigation and a similar situation is believed to be operating in the BPO system.

As discussed in the Introduction, the role of the suppressor cell in the control of the IgE response has been clearly demonstrated. Tada and his coworkers have positively identified the importance of suppressor cells in the normal reaginic response of rats (45-51). These workers established the presence of an antigen specific suppressor T cell that results in the early termination of most reaginic responses in the rat. It was only possible to establish a longlasting reaginic response in the rat when the normal T cell population was diminished by treatments such as adult thymectomy and splenectomy, sublethal X-irradiation and treatments with immunosuppressive agents.

Suppressor T cells have been found in the urea-denatured ovalbumin system studied by Takatsu and Ishizaka as well (41-44). These workers found that an intravenous injection of UD-OA suppressed an animal's response to the native ovalbumin molecule. The suppression was found to be an active process

mediated by splenic T cells and to be specific for the ovalbumin molecule.

Also, other work in this laboratory using protein-MγG conjugates has produced evidence for the involvement of suppressor cells in the control of the reaginic response (64). The MγG-protein conjugates used to establish tolerance were found to produce suppressor cells as the mode of tolerance induction. As well, conjugates of PEG and certain proteins (OA or RAG) were found to result in a tolerance in the animals, due to the presence of suppressor cells (65-66).

The system developed by Kishimoto and his coworkers also produced suppressor cells (67-70). A strong suppressive effect on the reaginic response was produced when mice were treated with DNP-coupled Mycobacterium (DNP-Tbc). It was found that the IgG response was not affected but the anti-DNP IgE response was greatly depressed. The suppressor cells appeared to be IgE class specific T cells and produced a soluble factor that was responsible for the suppressor activity. This factor was shown not to be antigen specific once it was produced, even though the induction was specific and stimulation with DNP was required for its production. The factor not only suppressed the anti-DNP response, but also the anti-OA response of animals sensitized with DNP-OA.

In this work, suppressor cells have also been found to be operating. However, there are a number of differences be- this system and that studied by Kishimoto that should be examined. First, the suppression produced by the PVA conjugates was found to affect both the IgE and the hemagglutinating

antibodies (Table VIII), as well as the hemolytic plaque forming cell response (results to be reported elsewhere). Secondly, the suppressive effect produced by the treatment with DNP-PVA was highly hapten specific, not influencing the anti-carrier (anti-OA) response at all. This is in contrast to the suppression produced by the treatment with DNP-Tbc which was found to be IgE class specific, but not antigen specific.

Recently, work has also been done by von Specht et al. (75) using a conjugate of NIP with poly(N-vinylpyrrolidone) (PVP) as discussed in the Introduction. In this study, the workers demonstrated that both the primary and the secondary IgG and IgM anti-NIP responses were suppressed with the treatment with this conjugate. Preliminary collaborative work between von Specht and Lee's laboratories have indicated that a single dose of NIP-PVP greatly suppresses the anti-NIP IgE response as well. The presence of suppressor cells in mice which had received NIP-PVP was also demonstrated by adoptive cell transfer experiments (84).

Perhaps the most important aspect of the work completed on the PVA system thus far, has been the demonstration of the protective nature of the PVA conjugates against systemic anaphylaxis. In both the DNP-PVA and the BPO-PVA systems, the anti-hapten IgE response is highly suppressed and the animals do not display any symptoms of systemic anaphylaxis upon intravenous challenge with the respective multivalent hapten-protein conjugate (i.e. DNP_9-MγG or BPO_9-MγG). The protection afforded by treatment with either DNP-PVA or BPO-PVA conjugates is highly specific however, and treatment with these

compounds does not provide any protection against systemic anaphylaxis caused by an intravenous injection of OA. This aspect of the work is extremely important, as it indicates the relevancy of the system to the therapeutic treatment of allergy patients, particularly penicillin allergy patients. The conjugates with low epitope density, such as $BPO_{1.1}$-PVA_{10} and $BPO_{1.3}$-PVA_{14} would prove to be the most useful, as they display only limited allergenicity, as discussed earlier. Because of their limited allergenicity and their ability to abrogate an existing reaginic response, these conjugates could be used to treat human penicillin allergy patients without the risk of inducing systemic anaphylaxis, as is the case with some desensitization regimes currently in use. If these conjugates are proven to be as effective in suppressing IgE antibody production in humans as they are in mice, a tremendous advance in allergy treatment will have been accomplished.

CLAIMS TO ORIGINALITY

1. The water-soluble, synthetic polymer polyvinyl alcohol of molecular weight either 10,000 (PVA_{10}) or 14,000 (PVA_{14}) daltons, was used as a tolerogenic carrier molecule. Amino derivatives of both PVA_{10} and PVA_{14} were prepared using the cyanogen bromide activation.

2. Conjugates of these activated PVA-NH_2 molecules with either DNP or BPO haptens were prepared and spectrophotometrically characterized to determine the hapten epitope density on the PVA molecule.

3. The effect of the DNP-PVA and BPO-PVA conjugates on the immunological responses of BDF_1 mice was studied. The conjugates were found to be very immunospecific in their effect on the immune responses of the mice. The anti-hapten reaginic and hemagglutinating antibody response was completely suppressed while the anti-carrier response was not influenced at all.

4. Both the primary and the secondary anti-hapten responses of normal animals were suppressed when the tolerogenic conjugate was administered prior to the initial sensitization. An ongoing anti-hapten response could be abrogated also, by the administration of the tolerogen to the sensitized animals.

5. A conjugate with an epitope density of at least 1.0 was required to establish a state of complete, longlasting

unresponsiveness to the hapten. Conjugates with epitope densities close to 1.0 had low allergenicity and strong tolerogenic properties.

6. The unresponsive state could be transferred into syngeneic, X-irradiated recipients by a transfer of spleen cells from tolerized animals.

7. Tolerized spleen cells suppressed the normal responses of primed spleen cells when admixtures of the two types of cells were transferred into syngeneic, X-irradiated recipients.

8. The DNP-PVA and the BPO-PVA conjugates were capable of protecting sensitized animals against systemic anaphylaxis induced by intravenous challenge with the multivalent-hapten-protein conjugate. The protection provided was highly immunospecific, however, and provided no protection against anaphylaxis caused by intravenous challenge with the carrier protein.

REFERENCES

1. Grabar, P. 1976. The historical background of immunology. In Basic and Clinical Immunology. Edited by H.H. Fudenberg, D.P. Stites, J.L. Caldwell, J.V. Wells. Lange Medical Publications. Los Altos, California. P 3-14.

2. Roitt, Ivan. 1974. Essential Immunology. 2nd ed. Blackwell Scientific Publications, London.

3. Claman, H.N. and Chaperon, E.A. 1969. Immunologic complementation between thymus and marrow cells - A model for the two-cell theory of immunocompetence. Trans. Rev. 1:92.

4. Parrot, D.M.V., de Sousa, M.A.P. and East, J. 1966. Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. J. Exp. Med. 123:191.

5. Davies, A.J.S., Leuchars, E., Wallis, V. and Koller, P.C. 1966. The mitotic response of thymus-derived cells to antigenic stimulus. Transpl. 4:438.

6. Gershon, R.K., Wallis, V., Davies, A.J.S. and Leuchars, E. 1968. Inactivation of thymus cells after multiple injections of antigen. Nature 218:380.

7. Davies, A.J.S., Leuchars, E., Wallis, V., Marchant, R., and Elliot, E.V. 1967. The failure of thymus-derived cells to produce antibody. Transpl. 5:222.

8. Miller, J.F.A.P. and Mitchell, M. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. J. Exp. Med. 128:801.

9. Mitchell, G.F. and Miller, J.F.A.P. 1968. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. J. Exp. Med. 128:821.

10. Nossal, G.J.V., Cunningham, A., Mitchell, G.F. and Miller, J.F.A.P. 1968. Cell to cell interaction in the immune response. III. Chromosomal marker analysis of single antibody-forming cells in reconstituted irradiated or thymectomized mice. J. Exp. Med. 128:839.

11. Taylor, R.B. 1969. Cellular cooperation in the antibody response of mice to two serum albumins: Specific function of thymus cells. Transpl. Rev. 1:114.

12. Chiller, J.M., Habicht, G.S. and Weigle, W.O. 1970. Cellular sites of immunologic unresponsiveness. PNAS 65:551.

13. Miller, J.F.A.P. and Sprent, J. 1971. Cell to cell interaction in the immune response. IV. Contribution of thymus-derived cells and antibody-forming cell precursors to immunological memory. J. Exp. Med. 134:66.

14. Mitchison, N.A. 1969. Immunocompetent cell populations. In Immunological Tolerance. Edited by M. Landy and W. Braun, Academic Press, New York.

15. Boak, J.L., Mitchison, N.A. and Pattison, P.H. 1971. The carrier effect in the secondary response to hapten-protein conjugates. III. The anatomical distribution of helper cells and antibody-forming-cell-precursors. Eur. J. Immunol. 1:63.

16. Britton, S., Mitchison, N.A. and Rajewsky, K. 1971. The carrier effect in the secondary response to hapten-protein conjugates. IV. Uptake of antigen in vitro and failure to obtain

cooperative induction in vitro. Eur. J. Immunol. 1:65.

17. Mitchison, N.A. 1971. The carrier effect in the secondary response to hapten-protein conjugates. V. Use of anti-lymphocyte serum to deplete animals of helper cells. Eur. J. Immunol. 1:68.

18. Rajewsky, K., Schirrmacher, V., Nase, S. and Jerne, N.K. 1969. The requirement of more than one antigenic determinant for immunogenicity. J. Exp. Med. 129:1131.

19. Katz, D.H., Paul, W.E., Goidl, E.A. and Benacerraf, B. 1970. Carrier function in anti-hapten responses. I. Enhancement of primary and secondary anti-hapten antibody responses by carrier preimmunization. J. Exp. Med. 132:261.

20. Paul, W.E., Katz, D.H., Goidl, E.A. and Benacerraf, B. 1970. Carrier function in anti-hapten responses. II. Specific properties of carrier cells capable of enhancing anti-hapten antibody responses. J. Exp. Med. 132:283.

21. Ishizaka, K. and Okudaira, H. 1972. Reaginic antibody formation in the mouse. I. Antibody-mediated suppression of reaginic antibody formation. J. Immunol. 109:84.

22. Ishizaka, K. and Okudaira, H. 1973. Reaginic antibody formation in the mouse. II. Enhancement and suppression of anti-hapten antibody formation by priming with carrier. J. Immunol. 110:1067.

23. Okudaira, H. and Ishizaka, K. 1973. Reaginic antibody formation in the mouse. III.Collaboration between hapten-specific memory cells and carrier-specific helper cells for secondary anti-hapten antibody formation. J. Immunol. 111:1420.

24. Hamaoka, T., Newburger, P.E., Katz, D.H. and Benacerraf, B. 1974. Hapten specific IgE antibody responses in mice. III.

Establishment of parameters for generation of helper T cell function regulating the primary and secondary responses of IgE and IgG B lymphocytes. J. Immunol. 113:958.

25. Hamaoka, T., Katz, D.H. and Benacerraf, B. 1972. Radioresistance of carrier-specific helper thymus-derived lymphocytes in mice. PNAS (USA) 69:3453.

26. Hamaoka, T., Katz, D.H., Block, K.J. and Benacerraf, B. 1973. Hapten-specific IgE antibody responses in mice. I. Secondary IgE responses in irradiated recipients of syngeneic primed spleen cells. J. Exp. Med. 138:306.

27. Hamaoka, T., Katz, D.H. and Benacerraf, B. 1973. Hapten-specific IgE antibody responses in mice. II. Cooperative interaction between adoptively transferred T and B lymphocytes in the development of IgE. J. Exp. Med. 138: 538.

28. Havas, H.F. 1969. The effect of the carrier protein on the immune response and on the induction of tolerance in mice to the 2,4-dinitrophenyl determinant. Immunology 17:819.

29. Golan, D.T. and Borel, Y. 1971. Nonantigenicity and immunogenic tolerance: The role of the carrier in the induction of tolerance to the hapten. J. Exp. Med. 134:1046.

30. Hamilton, J.A. and Miller, J.F.A.P. 1974. Hapten-specific tolerance in mice. I. Induciton with hapten-coupled syngeneic erythrocytes. Eur. J. Immunol. 4:261.

31. Hamilton, J.A., Miller, J.F.A.P. and Kettman, J. 1974. Hapten-specific tolerance in mice. II. Adoptive transfer studies and evidence for unresponsiveness in the B cells. Eur. J. Immunol. 4:268.

32. Katz, D.H., Davie, J.M., Paul, W.E. and Benacerraf, B. 1971. Carrier function in anti-hapten antibody responses. IV. Experimental conditions for the induction of hapten-specific tolerance on the stimulation of anti-hapten anamnestic responses by "non-immunogenic" hapten polypeptide conjugates. J. Exp. Med. 134:201.

33. Katz, D.H., Hamaoka, T. and Benacerraf, B. 1972. Immunologic tolerance in bone-marrow-derived lymphocytes. I. Evidence for an intracellular mechanism of activation of hapten-specific precursors of antibody-forming cells. J. Exp. Med. 136:1404.

34. Katz, D.H., Hamaoka, T. and Benacerraf, B. 1973. Induction of immunological tolerance in bone-marrow-derived lymphocytes of IgE antibody class. PNAS (USA) 70:2776.

35. Okumura, K. and Tada, T. 1971. Regulation of homocytotropic antibody formation in the rat. III. Effect of thymectomy and splenectomy. J. Immunol. 106:1019.

36. Michael, J.G. and Bernstein, I.L. 1973. Thymus dependence of reaginic antibody formation in mice. J. Immunol. 111:1600.

37. Michael, J.G. and Bernstein, I.L. 1974. Effect of thymocytes on reaginic antibody formation in mice. J. Allergy Clin. Immunol. 53:67.

38. Watanabe, N., Kojima, S. and Ovary, Z. 1976. Suppression of IgE antibody production in SJL mice. I. Nonspecific suppressor T cells. J. Exp. Med. 143:833.

39. Chiorazzi, N., Fox, D.A. and Katz, D.H. 1976. Hapten-specific IgE antibody response in mice. VI. Selective enhancement of IgE antibody production by low doses of X-irradiation and by cyclophosphamide. J. Immunol. 117:1629.

40. Chiorazzi, N., Fox, D.A. and Katz, D.H. 1977. Hapten-specific IgE antibody responses in mice. VII. Conversion of IgE non-responder strains to IgE responders by elimination of suppressor T cell activity. J. Immunol. 118:48.

41. Takatsu, K. and Ishizaka, K. 1975. Reaginic antibody formation in the mouse. IV. Suppression of IgE and IgG antibody responses to ovalbumin following the administration of high doses of urea-denatured antigen. Cell. Immunol. 20:276.

42. Takatsu, K. and Ishizaka, K. 1975. Regulation of anti-ovalbumin (OA) IgE and IgG antibody responses in mouse by administration of urea-denatured OA. Fed. Proc. 34:1000 (Abs. 4425).

43. Takatsu, K. and Ishizaka, K. 1976. Reaginic antibody formation in the mouse. VII. Induction of suppressor T cells for IgE and IgG antibody responses. J. Immunol. 116:1257.

44. Takatsu, K. and Ishizaka, K. 1977. Reaginic antibody formation in the mouse. IX. Enhancement of suppressor and helper cells activities of primed spleen cells. J. Immunol. 118:151.

45. Tada, T. 1978. Cellular interactions involved in the initiation and suppression of IgE synthesis in rats, Chap. 5. In Immediate Hypersensitivity - Modern Concepts and Developments. Edited by M.K. Bach. Marcel Dekker, Inc., New York. P 101-126.

46. Tada, T., Taniguchi, M. and Okumura, K. 1971. Regulation of homocytotropic antibody formation in the rat. II. Effect of X-irradiation. J. Immunol. 106:1012.

47. Tada, T. and Okumura, K. 1971. Regulation of homocytotropic antibody formation in the rat. I. Feedback regulation by passively administered antibody. J. Immunol. 106:1002.

48. Taniguchi, M. and Tada, T. 1971. Regulation of homocytotropic antibody formation in the rat. IV. Effect of various immunosuppressive drugs. J. Immunol. 107:579.

49. Okumura, K., Tada, T. and Ochiai, T. 1974. Effect of anti-thymocyte serum on reaginic antibody formation in the rat. Immunology 26:257.

50. Okumura, K. and Tada, T. 1971. Regulation of homocytotropic antibody formation in the rat. VI. Inhibitory effect of thymocytes on the homocytotropic antibody response. J. Immunol. 107:1682.

51. Tada, T., Okumura, K. and Taniguchi, M. 1972. Regulation of homocytotropic antibody formation in the rat. VII. Carrier function in the anti-hapten homocytotropic antibody response. J. Immunol. 108:1535.

52. Tada, T., Okumura, K. and Taniguchi, M. 1973. Regulation of homocytotropic antibody formation in the rat. VIII. An antigen-specific T cell factor that regulates anti-hapten homocytotropic antibody response. J. Immunol. 111:952.

53. Okumura, K. and Tada, T. 1974. Regulation of homocytotropic antibody formation in the rat. IX. Further characterization of the antigen-specific inhibitory T cell factor in hapten-specific homocytotropic antibody response. J. Immunol. 112:783.

54. Takemori, T. and Tada, T. 1974. Selective roles of thymus-derived lymphocytes in the antibody response. II. Preferential suppression of high-affinity antibody-forming cells by carrier-primed suppressor T cells. J. Exp. Med. 140:253.

55. Takemori, T. and Tada, T. 1975. Properties of antigen-specific suppressive T cell factor in the regulation of antibody response of the mouse. I. In vivo activity and immunochemical characterizations. J. Exp. Med. 142:1241.

56. Taniguchi, M., Hayakawa, K. and Tada, T. 1976. Properties of antigen-specific suppressive T cell factor in the regulation of antibody response of the mouse. II. In vitro activity and evidence for the I region gene product. J. Immunol. 116: 542.

57. Taniguchi, M., Tada, T. and Tokuhisa, T. 1976. Properties of the antigen-specific suppressive T cell factor in the regulation of antibody response of the mouse. III. Dual gene control of the T-cell-mediated suppression of the antibody response. J. Exp. Med. 144:20.

58. Tada, T., Taniguchi, M. and David, C.S. 1976. Suppressive and enhancing T-cell factors as I-region gene products: Properties and the subregion assignment. Cold Spring Harbour Symp. Quant. Biol. 41:119.

59. Tada, T., Takemori, T., Tokuhisa, T., Taniguchi, M. and Okumura, K. 1976. Suppressor T cells in allergy. Allergy and Clin. Immunol. Proceedings of IX International Congress of Allergology:95.

60. Lee, W.Y. and Sehon, A.H. 1975. Suppression of reaginic antibody formation. I. Induction of hapten-specific tolerance. J. Immunol. 114:829.

61. Lee, W.Y. and Sehon, A.H. 1975 Suppression of reaginic antibody formation. II. The use of adoptive transfer systems for the study of immunologic unresponsiveness. J. Immunol. 114:837.

62. Sehon, A.H. and Lee, W.Y. 1976. Specific immunosuppression of reaginic antibody formation. Allergy and Clin. Immunol. Proceedings of IX International Congress of Allergology :85.

63. Lee, W.Y. and Sehon, A.H. 1976. Suppression of reaginic antibody formation. IV. Suppression of reaginic antibodies to penicillin in the mouse. J. Immunol. 117:927.

64. Lee, W.Y. and Sehon, A.H. 1978. Suppression of reaginic antibodies. Immunol. Rev. 41:200.

65. Lee, W.Y. and Sehon, A.H. 1978. Suppression of reaginic antibodies with modified allergens. I. Reduction in allergenicity of protein allergens by conjugation to polyethylene glycol. Int. Arch. Allergy Appl. Immunol. 56:159.

66. Lee, W.Y. and Sehon, A.H. 1978. Suppression of reaginic antibodies with modified allergens. II. Abrogation of reaginic antibodies with allergens conjugated to polyethylene glycol. Int. Arch. Allergy Appl. Immunol. 56:193.

67. Kishimoto, T., Hirai, Y., Suemura, M. and Yamamura, Y. 1976. Regulation of antibody response in different immunoglobulin classes. I. Selective suppression of anti-DNP IgE antibody response by pre-administration of DNP-coupled Mycobacterium. J. Immunol. 117:396.

68. Suemura, M., Kishimoto, T., Hirai, Y. and Yamamura, Y. 1977. Regulation of antibody response in different immunoglobulin classes. III. In vitro demonstration of "IgE class-specific" suppressor functions of DNP-Mycobacterium-primed T cells and the soluble factor released from these cells. J. Immunol. 119:149.

69. Kishimoto, T., Hirai, Y., Suemura, M., Nakanishi, K. and Yamamura, Y. 1978. Regulation of antibody response in different immunoglobulin classes. IV. Properties and functions of "IgE class-specific" suppressor factor(s) released from DNP-Mycobacterium-primed T cells. J. Immunol. 121:2106.

70. Watanabe, N., Kimoto, M., Maruyama, S., Kishimoto, T. and Yamamura, Y. 1978. Regulation of antibody response in different immunoglobulin classes. V. Establishment of T hybrid cell line secreting IgE class-specific suppressor factor. J. Immunol. 121:2113.

71. Moreno, C., Hale, C. and Hewett, R. 1978. The use of hapten-polysaccharide conjugates for the induction of B cell tolerance involving IgE responses. I. Preparation, characterization and specific tolerogenic activity of penicilloyl-substituted levans affecting IgE responses in normal and sensitized mice. Clin. Exp. Immunol. 31:499.

72. Desaymard, C. 1977. Role of epitope density in the induction of immunity and tolerance with thymus-independent antigens. IV. Selective tolerance of IgE responses by DNP-levan conjugates. Eur. J. Immunol. 7:646.

73. Watanabe, N., Kojima, S. and Ovary, Z. 1977. Tolerizing effect of DNP-Ficoll on IgE antibody production. J. Immunol. 118:251.

74. Chiorazzi, N., Eshlar, Z. and Katz, D.H. 1976. Induction of immunological tolerance to the major antigenic determinant of penicillin: A therapeutic approach to penicillin allergy. PNAS (USA) 73:2091.

75. von Specht, B.U., Milgrom, L. and Segal, S. 1978. Induction of a stable hapten-specific immunosuppression by a hapten conjugated to poly(N-vinylpyrrolidone)(PVP). Clin. Exp. Immunol. 33:292.

76. Diner, U.E., Kinimoto, D. and Diener, E. 1979. Carboxymethyl cellulose, a nonimmunogenic hapten carrier with tolerogenic properties. J. Immunol. 122:1886.

77. Parker, C.W., de Weck, A.L. and Eisen, H.N. 1962. The preparation and some properties of penicillenic acid derivatives relevant to penicillin hypersensitivity. J. Exp. Med. 115:803.

78. Levine, B.B. and Vas, N.M. 1970. Effect of combinations of inbred strains, antigen and antigen dose on immune responsiveness and reagin production in the mouse. Int. Arch. Allergy 39:156.

79. Avrameas, S., Taudou, B. and Chiulon, S. 1969. Glutaraldehyde, cyanuric chloride and tetraazotized O-dianisidine as coupling reagents in passive hemagglutination test. Immunochem. 6: 67.

80. Mossman, H., Meyer-Delius, M., Vortisch, U., Kickhofen, B. and Hammer, D.K. 1974. Experimental studies on the bridging hypothesis of anaphylaxis. Haptenic determinants required to elicit immediate-type sensitization with reagins of different specificity. J. Exp. Med. 140:1468.

81. Stanworth, D.R. 1973. Immediate hypersensitivity. In Frontiers of Biology Series. Vol. 28. North Holland/ Elsevier, Amsterdam.

82. Levine, B.B. 1965. The nature of the antigen-antibody complexes which initiate anaphylactic reactions. I. A quantitative comparison of the abilities of nontoxic univalent, toxic univalent, divalent and multivalent benzyl penicilloyl haptens to evoke passive cutaneous anaphylaxis in the guinea pig. J. Immunol. 94:111.

83. Borel, Y., Kilham, L. Hyslop, N. and Borel, H. 1976. Isologous IgG induced tolerance to benzylpenicilloyl. Nature 261:50.

84. Lee, W.Y., von Specht, B.U. and Sehon, A.H. 1979. Suppression of anti-NIP reaginic antibody response with NIP-conjugated poly(N-vinylpyrrolidone). (In preparation).