SUPPRESSION OF THE MURINE ANTIBODY RESPONSE TO THE TRIMELLITYL (TM) GROUP WITH CONJUGATES OF TM AND POLYVINYL ALCOHOL

BY

BING-YUAN WEI

A thesis submitted to the Faculty of Graduate studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Immunology
University of Manitoba
Winnipeg, Manitoba
May, 1986

ISBN 0-315-37253-2

SUPPRESSION OF THE MURINE ANTIBODY RESPONSE TO THE TRIMELLITYL (TM) GROUP WITH CONJUGATES OF TM AND POLYVINYL ALCOHOL

BY

BIN-YUAN WEI

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

DOCTOR OF PHILOSOPHY

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

First of all, I should like to acknowledge the consistent support and encouragement of my parents, without which I may not have been able to accomplish this task.

I also wish to express my deep gratitude to Professor Alec Sehon, Head of the Department of Immunology, for his guidance throughout this investigation and for his constructive criticisms in the preparation of this thesis.

To Dr. F.T. Kisil, Dr. A. Froese, Dr. R.J. Schwenk and Dr. B.G. Carter, I am grateful for their helpful discussions and encouragement.

In particular, I wish to extend my thanks to Dr. Valerie Holford-Strevens for her valuable advice in planning the experiments and for the preparation of the thesis.

Finally, I wish to express my gratitude to the Ministry of Education of the People's Republic of China and to the University of Manitoba for the award of graduate fellowships which enabled me to come to Canada and to pursue this study.

This investigation was supported by funds granted in support of Professor Sehon's research program by the Medical Research Council of Canada and the National Institutes of Health of the U.S.A.

List of abbreviations:

Asc	Ascaris extract
BDF1	$B_6D_2F_1$
Bp	*Bordetella pertussis*
BPO	benzylpenicilloy
BSA	bovine serum albumin
BUdR	5' bromouridine deoxyriboside
C.P.M.	counts per minute
DGL	copolymer of D-glutamic acid and D-lysine
DMF	N,N-dimethyl formamide
DNP	dinitrophenyl
EDCI	1-ethyl-3(3-dimethylaminopropyl) carbodiimide HCl
FCA	Freund's complete adjuvant
FCS	fetal calf serum
GAME	goat anti-mouse IgE
KLH	keyhole limpet hemocyanin
LRSS	late respiratory systemic syndrome
MGG	mouse globulin
mPEG	monomethoxy-polyethylene glycol
Nb	*Nippostrongylus Brasiliensis*
NP	4-hydroxyl-3-nitrophenyl acetyl
OA	ovalbumin
OD	optical density
PBS	phosphate buffered saline
PCA	passive cutaneous anaphylaxis
PEG	polyethylene glycol

PFC	plaque forming cell
PWM	pokeweed mitogen
SAFab	sheep anti-mouse Fab
SRBC	sheep red blood cells
SRS-A	slow reacting substance of anaphylaxis
TDI	toluene diisocyanate
Th	helper T cells
TM	trimellytyl
TMA	trimellitic anhydride
Ts	suppressor T cells

ABSTRACT

Trimellitic anhydride (TMA) is widely used in the plastics industry and is considered to be the causative agent of several occupational respiratory disorders. IgE antibodies specific for the trimellityl group (TM) have been implicated in at least one of these syndromes, i.e.,rhinitis-asthma.

Since conjugates of different haptenic groups with polyvinyl alcohol (PVA) were previously shown in this Laboratory to suppress the corresponding anti-hapten IgE responses in mice, the suppressive effects of several TM-PVA conjugates on the murine anti-TM antibody response were investigated in this study. Using PVA preparations with average molecular weights of 3,000 and 14,000 daltons , referred to as PVA_3 and PVA_{14} , respectively, the TM group was coupled directly or via a hexanediamine spacer to PVA. Treatment of mice, either prior to or following immunization with TM-OA conjugates (OA=ovalbumin) in aluminum hydroxide resulted in a marked reduction of anti-TM antibodies of the IgE and other immunoglobulin classes. The suppressive capacity of the TM-PVA conjugates was dose- and epitope density-dependent.

The immunosuppressed state of spleen cells from TM-PVA treated mice was maintained following their transfer into irradiated syngeneic recipients and was not reversed by incubation of the cells in tolerogen-free medium prior to adoptive transfer. The spleen cells of TM-OA primed mice were also tolerized with respect to TM on incubation with TM-PVA. The persistent murine IgE response is known to be radiation resistant . However, a single dose of 1 mg of TM_7-PVA_{14}, injected i.p. 18 weeks after primary immunization, almost completely

suppressed the radiation resistant anti-TM IgE response for at least 6 weeks.

Investigation of the *in vivo* distribution of two hapten-PVA conjugates, i.e.,TM_7-PVA_{14} and $N^{125}IP$-PVA_{14} conjugates revealed that these conjugates disappeared rapidly from circulation. However, their presence in different organs, such as liver and kidney, could be demonstrated up to 100 days.

By contrast to other hapten-PVA systems, no evidence was adduced in support of the participation of transferable TM-specific suppressor T (Ts) cells. Hence, it was concluded that the mechanism of tolerance induced by TM-PVA involved primarily, if not exclusively, the inactivation of B cells by the blockade of their haptenic receptors.

SCOPE OF THE PRESENT RESEARCH

Industrial allergy and occupational asthma have received more attention in recent years since an increasing number of industrial chemicals are encountered in the work place. A variety of low molecular weight chemicals, including both organic and inorganic, e.g., nickel salts, antibiotics, trimellitic anhydride (TMA), toluene diisocyanate (TDI), phthalic anhydride, have been shown to cause allergy in sensitive patients.

Trimellitic anhydride, a chemical widely used in the plastics industry, has been shown to cause a spectrum of respiratory syndromes which are attributed to the specific IgE and IgG antibodies detected in the serum of exposed workers. The abrogation of these antibodies may be expected to lead at least to the alleviation of the symptoms, if not to the total cure of their disease conditions.

The primary aim of this study was to test the capacity of a series of TM-PVA conjugates, varying in molecular composition, to suppress the anti-TM antibody response in a mouse model system. Once their immunosuppressogenicity was established,the underlying mechanism of their downregulating effect on antibody response was also investigated.

In the first section is given a selectivey limited LITERATURE REVIEW of the cellular basis of IgE production and regulation , as well as of the general mechanisms of immune tolerance, with special reference to hapten-specific tolerance. A brief reference is also made to industrial allergies induced by TMA.

In the section entitled MATERIALS AND METHODS, the preparation of different conjugates and the pertinent materials and methods used in

this study are described.

The RESULTS of the study are described in four separate chapters dealing with different but related aspects, i.e.:

(i) In Chapter 1 , the suppressive effect of a series of TM-PVA conjugates was tested on the induction and on an ongoing anti-TM response, and the capacity of these conjugates to reverse the state of hypersensitivity of presensitized mice by protecting them from an anaphylactic shock was demonstrated.

(ii) Chapter 2 deals with the radio-resistant IgE response and its sensitivity to the treatment of TM-PVA. The possible mechanism of the suppression of the protracted, radio-resistant IgE response by the TM-PVA conjugates was investigated and discussed.

(iii) In Chapter 3 , the *in vivo* distribution of two different hapten-PVA conjugates, i.e., TM_7-PVA_{14} and $N^{125}IP$-PVA_{14}, was established and the relevance of their localization with respect to their immunosuppressive capacity was discussed.

(iv) In Chapter IV, the mechanism of immunosuppression caused by TM-PVA was investigated and it was concluded that treatment with TM-PVA resulted in the tolerization of B cells without any demonstrable participation of Ts cells.

Finally, in the section entitled GENERAL DISCUSSIONS an attempt was made to integrate all the results obtained in this study into a coherent survey and to indicate the possible "contribution to knowledge" of this investigation.

LITERATURE REVIEW

In 1921 Prausnitz and Kuestner first described the presence of a reaginic factor (skin-sensitizing antibody) in the serum of allergic individuals (1), by the passive cutaneous transfer of the hypersensititivy to normal individuals. The Ishizakas (1) demonstrated in 1966 that reagins belonged to a distinct class of immunoglobulins, which they named as IgE; and this represents the cornerstone of the modern era of investigation of the mechanism underlying cellular interaction involved in the synthesis of IgE and its regulation, as well as the molecular and pharmacological aspects of the inflammatory reactions attributed to IgE.

The most important biologic activity of IgE is its ability to sensitize homologous tissues, a characteristic which has been used for its bioassay. In recent years this unique property of IgE has been demonstrated to be due to the tenacious attachment of its Fcϵ fragment to the corresponding Fcϵ receptors of mast cells and basophils. After challenging IgE sensitized cells with multivalent antigen or anti-IgE antibodies, Fcϵ receptors become crosslinked and the vasoactive mediators are released, thus leading to an increase in secretions, contraction of smooth muscles and chemotaxis for eosinophils (1).

However, it should be noted that in various animal species, two classes of homocytotropic antibodies that can induce immediate-type allergic skin reactions have been demonstrated; one belonging to the IgE class, and the other being a subclass of IgG (1).

1. Experimental system for IgE production

Using the Prausnitz-Kuestner tests, Sherman *et al* (2) demonstrated that reaginic antibody in hay fever patients persisted for a long period of time. Furthermore, quantitative measurement of serum IgE antibody in untreated ragweed-sensitive patients showed that the antibody level was persistent and that most patients showed a secondary IgE antibody response after the ragweed season (3). Since the mean catabolic rate of human IgE is extremely fast with an average half life of 2.3 days (18), the high and persistent level of IgE was attributed to its being produced continuously in these patients. However, early attempts to induce a persistent IgE antibody response in experimental animals met consistently with failure (1). Thus , rats and mice which were injected with protein antigens, with or without adjuvants developed a transient IgE antibody response ending within 3 to 4 weeks, and a booster injection of the same antigen failed to induce a secondary IgE response (31).

Finally in 1970, Levine and Vaz (4) succeeded in producing an mouse model for mimicking IgE production. They immunized several inbred strains of mice with a minute dose of antigen (0.1-1 μg) incorporated in aluminum hydroxide and obtained a primary IgE antibody response within 7-14 days. A second injection after 4 weeks resulted in a secondary IgE antibody response of high IgE titres. Furthermore, Vaz (5) obtained a persisting IgE antibody response for several months without a booster by injecting OA (0.1 μg) absorbed on aluminum hydroxide into the SW-55 strain of mice. Vaz *et al* (6) also

demonstrated that the IgE antibody response was controlled by Ir genes, which are closely linked to the H-2 histocompatibility gene complex. When a small dose of antigen (OA or ovomucoid) was given along with aluminum hydroxide, some strains of mice developed IgE and IgG antibody responses. High responsiveness to ovalbumin was shown to be linked with H-2b and H-2d alleles, and that to ovomucoid with H-2a and H-2k alleles. However, SJL mice (H-2s) were shown to be poor IgE responders to several different antigens at different doses, although they gave reasonable IgG responses to some of the antigens; the genetic control seems to be related to IgE response uniquely. Breeding experiments showed that the genetic control mechanisms was not linked to H-2 locus (7). Therefore, two genetic control mechanisms may determine the murine IgE response to a given antigen, one being linked to MHC gene complex, and the other to background genes (6,7).

Several authors have investigated the mechanism underlying the protracted IgE production. Peeters *et al* (8,9) discovered in this laboratory that spleen cells from OA-primed B6D2F1 (BDF1) mice produced an anti-OA IgE response in irradiated syngeneic recipients (600 rads) in the absence of further overt homologous antigen challenge and that the anti-OA IgE response persisted for at least 6 months. They also observed that the cells responsible for the protracted responses could be best demonstrated at least 4 weeks after antigen stimulation of the donors. The proliferation of both T and B lymphocytes in the recipients was essential for the transferred response, since treatment of these cell populations with either gamma irradiation (> 200 rads) or mitomycin C before transfer resulted in the

loss of their ability to mount an IgE response in the recipient mice. However, once IgE production reached a steady state in the recipients, it manifested resistance to high doses of irradiation. Similarly, Okudaira *et al* (10) established a persistent anti-DNP (DNP = 2,4-dinitrophenyl) IgE response in BDF1 mice and demonstrated that a lethal dose (1,000 rads) of irradiation did not affect the persistent IgE or IgG responses though it prevented the recruitment of B memory cells, as demonstrated by the lack of a secondary antibody response and deletion of splenic DNP-primed B memory cells as shown in an adoptive cell transfer experiment. Okudaira *et al* (10) expanded DNP-primed B memory cells by adoptive transfer of DNP-KLH (keyhole lympet hemocyanin) primed spleen cells into irradiated mice followed by challenge with homologous antigen. Culture of spleen cells of the recipients depleted of T cells, in the absence of exogeneous antigen, resulted in the continuous production of both anti-DNP IgE and IgG antibodies *in vitro*. The antibody production was radioresistant and continued for 14 days. They suggested that long-lasting antibody forming cells were responsible for the protracted antibody responses. Holt *et al* (11,12,13) reproduced the phenomenon both in mice and in BN rats, and showed resistance of this response to downregulation by Ts cells and that the long-lasting antibody producing cells in rats were localized in the bone marrow and draining lymph node cells.

Similar cells may account for the persistent IgE production in man. Thus, Okudaira *et al* (*14*) reported that peripheral blood lymphocytes (PBL) from atopic patients could secrete IgE antibodies in the absence of T cells and pokeweed mitogen (PWM) and that IgE antibody

production was not significantly affected by addition of T cells or PWM. Moreover, irradiation (1,000 R) decreased more than 50% of the IgE and IgG antibody producing ability of the cells only in 2 out of 8 cases. Further increase of the dose of irradiation to 12,000 R did not result in higher degree of suppresssion. It would, therefore, appear that PBL of atopic patients contain both radio-resistant and radio-sensitive subpopulations of B cells forming IgE antibodies.

2. Precursors of IgE-forming cells

The nature of surface immunoglobulins on murine B cells has been extensively investigated. Virgin B cells have been shown to bear surface IgM, and B cells bearing other isotypes are derived from these cells. It is generally accepted that lymphocytes bearing a given surface immunoglobulin isotype (sIgM, sIgA, or sIgE) are committed to the synthesis of the corresponding isotypes (15).

The development from IgM virgin cells to the IgM-IgE bearing cells seems to be a T-independent process, since neonatal thymectomy does not influence the appearance of these cells carrying both isotypes (16,17). The athymic nude mice were also found to have IgE-bearing cells in their spleens (17), but not to be capable of producing IgE (19).

To identify the precursors of IgE-forming cells, Suemura *et al* (20) established an *in vitro* system, in which immunoglobulin-forming cells could be developed from B lymphocytes . In their experiments, mesenteric lymph node (MLN) cells from either normal rats or rats infected with *Nippostrongylus Brasiliensis* (Nb) were incubated for 5 days with PWM, and the cells were then centrifuged and examined by

immunnofluorescence for their cytoplamic imunoglobulins. It was shown that a substantial number of IgM, IgE and IgG2a forming cells developed when cells from Nb-infected rats were stimulated with PWM. By the deletion of IgE bearing cells or IgM- bearing cells with corresponding antibodies, a marked decrease of the *in vitro* IgE response was observed. It was, therefore, concluded that the majority of the precursors of IgE forming cells were bearing both IgM and IgE. However, it was later demonstrated that the majority of B cells bore IgM and IgD on their surfaces (21) and more than 90% of IgE-bearing cells in both normal or Nb-infected rats had surface IgM (22). The deletion of IgD-bearing cells also resulted in a marked decrease of *in vitro* IgE-forming cell response (20). Thus, it seems that the majority of IgE-bearing cells also bear surface IgM and IgD, and that these cells are actually precursors of IgE-forming cells. The results of experiments by Bazin *et al* (23) which involved the injection of anti-δ antibodies into neonatal animals confirmed the above conclusions in that IgE responses were suppressed by this treatment.

As mentioned above, the process of differentiation of virgin B cells into ϵ-bearing cells is T-indepndent. However, these triple-Ig bearing cells, i.e., cells bearing μ, δ, and ϵ chains differentiate into IgE-forming cells through a T-dependent process, as demonstrated by the facts that (i) purified rat MLN B cells failed to respond to PWM in the above cell cultures; and (ii) when B cells of Nb-infected rats were mixed with T cells from normal or Nb-infected rats and stimulated with Nb antigen, IgE-forming cells formed only in the cell cultures with T cells from Nb-infected animals (21).

3. Requirement for T cells in IgE production

It is a well established fact that antibody responses to most antigens (T cell dependent antigens), require the collaboration of T cells and B cells; thus by the use of hapten-carrier conjugates it was shown that T cells are usually primed by the carrier determinants, whereas B cells are primed by the haptenic determinants (24). As mentioned earlier, the IgE antibody response was shown to be strongly T cell dependent by the following evidence.

3.1. Neonatal thymectomy abolishes the IgE antibody response. Similarly, congenitally athymic nude (nu/nu) mice fail to produce IgE antibody, although IgE-bearing cells are present in their spleens (25). However, with the supplement of thymocytes from nu/+ mice, nude mice are able to produce IgE antibodies (26).

3.2. It was shown by Okudaira *et al* (27) that DNP-Ascaris (Asc) immunized mice failed to produce a secondary anti-DNP IgE response after challenge with DNP-heterologous protein, such as DNP-OA. However, if these mice were given a supplemental immunizing dose of OA after DNP-Asc, they developed a secondary anti-DNP IgE antibody on subsequent stimulation with DNP-OA. These results further suggesst that DNP memory cells cooperate with OA-specific T helper (Th) cells to produce anti-DNP IgE antibody .

3.3. By the adoptive transfer technique, Hamaoka *et al* (28) clearly demonstrated T-B cell collaboration in IgE antibody production. They immunized two groups of mice with either DNP-KLH or Asc. After certain time intervals the spleen cells of each group of donors were

transferred to irradiated syngeneic recipients. Adoptively transferred DNP-KLH primed cells produced high levels of anti-DNP IgE in response to DNP-KLH but not to DNP-Asc. However, recipients of both DNP-KLH primed cells and Asc primed cells developed a strong secondary IgE and IgG anti-DNP response after challenge with DNP-Asc.

4. Regulation of IgE response by Ts cells

Gershon and Kondo (29) first showed the role of T cells in the regulation of antibody repsonse to sheep red blood cells (SRBC). When splenic cells of mice tolerant to SRBC were transferred to normal mice, the recipients were shown to be tolerant to SRBC. With respect to the IgE antibody response, Okumura and Tada (30) observed that adult thymectomy, splenectomy, or both, significantly enhanced IgE antibody responses of the rat to DNP-Asc administered in the presence of *Bordetella pertussis* (Bp). In their experiment, the deletion of these organs did not affect the formation of agglutinating antibodies, the effect being exclusively limited to the IgE antibody response. Interestingly, Okumura and Tada (30) observed that when rats were hyperimmunized with antigen in the presence Freund's complete adjuvant (FCA) no IgE was formed. If donors were repeatedly immunized with DNP-Asc or Asc in FCA and their lymphocytes or spleen cells were transferred into recipients which were producing high titres of IgE antibody, the anti-Asc IgE of the recipients dropped dramatically within two days of cell transfer. However, cells from donors immunized with only DNP-Asc or normal animals did not show any inhibitory effect. This carrier-specific inhibitory activity of spleen cells was

completely abolished when cells were treated with anti-thymocyte serum plus complement. These authors hypothesized originally that large number of Th cells negatively regulate the ongoing response, i.e., "too much help" was equivalent to a negative signal. It is now considered that the previous observation of the suppression of ongoing IgE response in the rats was probably due to another subpopulation of T cells, namely Ts cells (24).

Most strains of rats respond to appropriate antigens and adjuvant with a moderate and transient IgE response, and only brief secondary IgE responses are elicited in such animals (31). However, Tada *et al* (32) found that a sublethal dose of 400 R shortly before or after immunization with DNP-Asc plus Bp substantially enhanced the production and the duration of the anti-DNP IgE response, while the irradiation inhibited IgM and IgG responses.

Consistent with these observations were studies by Taniguchi and Tada (33) in which it was observed that 5-bromouridine deoxyriboside (BUdR), an inhibitor of DNA synthesis, markedly enhanced and prolonged the IgE responses when the drug was given on the same day of immunization or 2-5 days afterwards. Similarly, treatment with actinomycin D, an inhibitor of DNA-dependent RNA synthesis, resulted in accelerated and persistent IgE responses, provided the drug was given shortly after the immunization with DNP-Asc (Ascaris). Yet at the same time, the IgM and IgG responses were either depressed or abrogated. The administration of cyclophosphamide or cortisone prior to immunization resulted in a similar phenomena.

In subsequent experiments, Tada *et al* (34) showed that animals

preimmunized with Asc in FCA 1 month prior to immunization with DNP-Asc suppressed the production of anti-DNP IgE response. However, preimmunization with DNP-BSA in FCA failed to suppress the anti-DNP IgE response. The above results indicated that preimmunization with Asc in FCA resulted in the induction of Asc-specific Ts cells which were capable of suppressing the IgE response to DNP coupled to Asc.

In mice, similar observations were made. It was first observed by Hamaoka *et al* (35) that the magnitude of a secondary response obtained in irradiated mice by adoptive transfer was much stronger than that elicited in intact mice . Subsequently, Fox *et al* (36) and Chiorazzi *et al* (37) demonstrated that exposure to low dose of irradiation (50 to 200 R) of unprimed or primed A/J, BALB/c and CAF1 mice, substantially enhanced IgE responses in these animals, while IgG antibody responses were generally unaffected. Similar results were obtained in primed mice pretreated with cyclophosphamide (2 days prior to secondary challenge), although a considerable delay in kinetics of enhancement was demonstrated which was probably due to the initial B cell recovery from the effects of the drug (37).

Watanabe *et al* (7) found that SJL mice, a strain which is generally considered to be a low responder in terms of IgE production, could develop a strong IgE antibody response when exposed to 540 R X-irradiation 1 or 4 days after challenge with DNP-Nb. In these studies, the investigators determined that the enhanced and sustained IgE response was due to the elimination of radiosensitive, non-antigen specific Ts cells which exerted a relatively isotype specific action on IgE production.

In summary, the dissociation of Th and Ts activities between IgE and IgG antibody responses is observed under a variety of conditions: 1) The IgE antibody response is highly dependent upon the adjuvant employed, thus Bp and aluminum hydroxide generally favor IgE production, whereas repeated FCA injections resulted in the suppression of IgE without affecting the IgG antibody response; 2) Irradiation of mice with low dose of X-ray, or an injection of cyclophosphamide prior to or shortly after immunization selectively enhances the IgE response; 3) SJL mice fail to produce IgE responses in spite of substantial IgG response to the same antigen. Infection of human and rodents with nematode selectively enhances the IgE synthesis (1). All the above facts suggest that IgE antibody response is determined not only by antigen-specific helper and suppressor T cells, but also by cells and factors governing specific isotypic regulation.

In the past few years, several groups, especially the Ishizakas, concentrated on the possible mechanisms of this isotypic regulation of the IgE antibody response. They found that there are two T cell binding factors which have affinity for IgE (i.e., IgE binding factors) which either enhance or suppress IgE responses (38,39,40). In spite of the importance of the conclusions of the Ishizakas' results, these will not be discussed in detail here, since their systems are not directly relevant to the study undertaken by the author.

5. Immune Tolerance

Immune tolerance can be defined as a specific suppression of the immune response induced by previous exposure to the antigen. Extensive studies have shown that factors, such as the dose of antigen, its physical properties , the physiological state and genetic background of the host influence the degree, duration and cellular basis of the tolerance state (41).

Detailed studies by Mitchison (42) indicated that tolerance to a protein molecule in adult mice could be induced in two distinct dose ranges: the high and low zone tolerance. Weigle and co-workers (43) clarified the cellular basis for these two zones of tolerance: low zone tolerance reflects a specific unresponsiveness in the Th population, whereas high zone tolerance reflects a specific unresponsiveness in both Th and B cells. However, for T-independent antigens these two distinct zones tolerance were not observed , and in particular low zone of tolerance is not a universal phenomenon (44,45).

Certain routes of antigen presentation tend to favor tolerance rather than immunization. For example, intravenous administration of chemically active haptens (48) or administration of proteins (e.g. OA) via the stomach favors the induction of tolerance (46,47).

It is well known that it is fairly easy to induce tolerance with soluble antigens, especially after their being subjected to ultracentrifugation for removal of any aggregates. Other physical properties, such as molecular weight and epitope density may also affect the tolerance induction.

In general, three major mechanisms (receptor blockade; clonal abortion, deletion or anergy and Ts cells) have been involved to explain the induction of tolerance. The specific mechanism responsible for tolerance to a given antigen may depend on the nature of the tolerogen, the procedure employed to induce tolerance and the nature of the host, etc (41).

Clonal deletion, also referred to as clonal abortion, implies that immature lymphocytes pass through a certain stage in their development during which interaction of the antigen with receptors on the cells may lead to their deletion . If clonal deletion were the only mechanism for tolerance, one would expect that there would be no antigen-binding cells present in tolerant animals. However, the results of various investigations indicates that in many instances the antigen-binding cells are not eliminated in the tolerant state (49). Recently, Nossal *et al* (50,51,52) have indicated that the corresponding B cells are not necessarily deleted in the tolerized animals and that they persist in the form of tolerized B cells, i.e., they are functionally inactivated, and therefore referred to this form of tolerance as B cell anergy rather than B cell deletion.

One of the earliest ideas about tolerance was Burnet's notion that lymphoid cells early in differention would be rendered tolerant more easily than in adults (41,51). Indeed, there is substantial evidence that B cells at different stages of maturation display markedly different sensitivities to negative signalling (50,52,53). As has been referred to earlier, B cells are derived from proliferating pre-B cells and after mitotic cycles they develop into small non-dividing

lymphocytes lacking surface Ig receptors. Then in a non-mitotic maturation phase, they first develop IgM, and later IgM+IgD (57).

Cooper *et al* (53) have shown in their elegant experiments that when anti-μ antibodies were injected into neonatal mice, B cell maturation could be prevented and these mice did not produce immunoglobulin of any classes . However, these animals possessed pre-B cells, since such lymphocytes cultured in the absence of anti-μ antibody became surface immunoglobulin positive. Experimental evidence has suggested that in a significant portion of immature B cells the surface immunoglobulin does not recover on the membrane after crosslinking by anti-μ antibodies, while in mature B cells the loss of membrane immunoglobulin is readily reversible.

Nossal *et al* (51,57) have indicated that cells encountering antigen in the stage of the appearance of first Ig receptors are most sensitive to tolerance, i.e., the stage when the pre-B cells become B cells. The more mature the B cells, the more difficult the induction of B cell tolerance. However, even for antibody forming cells, antibody production can be shut off by high concentration of antigen (54,55,56).

In fact, activated B cell blasts and early antibody forming cells including many plasma cells still bear surface Ig, indicating that these cells are probably still under the regulatory control of antigen (57). Schrader & Nossal (52,54) reported that interaction of antibody forming cells with multivalent antigens resulted in the profound decrease of the secretory rate of immunoglobulins from these specific cells. Abbas *et al* (55) independently showed that antibody production

by hybridoma cell lines can also be inhibited by the appropriate multivalent antigen. Thus, utilizing a hybridoma cell line, Boyd *et al* (56) showed that the decrease of antibody secretion was due to the reduced Ig synthesis. The appropriate multivalent antigen causing blockade aggregated on cell surface, and clearance of cell-associated antigen correlated with recovery of the blockade.

Several models of B cell tolerance have been investigated in which the unresponsiveness was readily reversed after transferring the cells into antigen-free enviroment or after digesting the antigen from the cell surface. The tolerance is believed to be due to the occupance of the receptors by the tolerogen. The reversal of tolerance in this instance is usually accompanied by shedding the antigen from the cell membrane (24).

Jerne's postulate (41) that the immune system operates as an extended network of idiotypes and anti-idiotypes has been experimentally confirmed in many laboratories. This network operates presumably at the T cell, B cell and antibody levels. It is known that anti-idiotypic antibody or Ts cells can depress the production of antibodies bearing the corresponding idiotype, i.e., downregulate the immune response (41).

In general, the mechanism regulating the IgE antibody response is similar to that responsible for the IgG response. However, the regulation of the IgE isotype has its own unique features. As discussed earlier, the precursors of IgE forming cells have been shown to be both sIgE positive and sIgM positive. The expression of sIgE on sIgM and sIgM/sIgD positive virgin B cells does not require the help

of T cells or stimulation of antigen. Hence, the expression of sIgE on B cells is T-independent and antigen-independent. It occurs in the fetal or neonatal period. Once IgE is expressed on the surface of B cells, these cells are then committed for the production of IgE. However, maturation of these IgE positive B cells into IgE antibody forming cells stilll requires cooperation of Th cells, and this process can be regulated by Ts cells. Since the expression of IgE on the cell surface cannot be suppressed, the major approaches to suppress IgE responses involve the inactivation of the precursor B cells or the activation of Ts cells.

6. Induction of hapten-specific tolerance

The current hyposensitization methods for IgE-mediated allergies include: 1) the administration of certain drugs, i.e.,antihistamines and cromoglycates which may inhibit the release of mediators from mast cells or basophils or drugs, such as β-adrenergic drugs which may antagonize the effects of the released mediators; 2) hyposensitization therapy which consisits of a series of injections of the corresponding antigen in minute doses over a prolonged period of years. However, hyposensitization therapy does not lead always to the reduction of specific IgE and, hence of the sensitivity of mast cells and basophils. Moreover, this form of treatment is also associated with the risk of inducing systemic reactions. Therefore, many investigators have attempted to decrease the allergenicity of the allergenic preparations by chemical modification , and more recently attempts have been made to convert the allergens from an immunogenic form to a tolerogenic one.

It was repeatedly shown that when an antigen or polymer, that is "non-immunogenic" by itself, is used as a carrier for haptenic determinants, the induction of hapten-specific tolerance was achieved. In this connection, several nonimmunogenic carriers have been studied: e.g., 1) syngeneic immunoglobulins (58,59,60,61), 2) syngeneic cells, including those bearing Ia antigens (63,65) or red cells which do not bear Ia antigens (66), 3) some foreign material which lacks immunogenicity or to which the animal is genetically unresponsive, such as the copolymer of D-glutamic acid and D-lysine (DGL) (24), monomethoxy polyethylene glycol (mPEG) (67), polyvinyl alcohol (PVA) (67), dextrans (80,81), polyacrylamide (83,84,85,86) , and certain nonmetabolizable polysaccharides (87,88).

Borel *et al* (58) extensively studied the tolerance induced by hapten (DNP) conjugates of isologous IgG. In their studies, it was shown that DNP-MGG (MGG = mouse globulin) persisted on the surface of lymphocytes, whereas DNP-protein conjugates did not (58). The spleen cells from mice rendered tolerant with DNP-MGG remained tolerant when transfered to irradiated recipients. However, 24 hours of *in vitro* incubation prior to transfer could reverse the tolerant state. Radioautographic studies showed that after incubation *in vitro* the antigen-binding cells with free receptors for DNP significantly increased. These data were interpreted as indicating that the mechanism of tolerance induction by DNP-MGG involved receptor blockade. They further demonstrated that haptenated $F(ab')_2$ were ineffective as tolerogens, suggesting the involvement of Fc receptors in the negative signalling. Pike *et al* (59) and Waldschmidt *et al* (60) confirmed the

above findings by showing that the removal of Fc portion greatly lowered the ability of haptenated human gamma globulin to induce tolerance. However, by increasing the hapten density, they were able to increase the tolerogenicity of hapten-$F(ab')_2$. In this laboratory, Lee *et al* (61) showed that the anti-DNP IgE response which was inducible by DNP_3-OA could be abrogated by i.v. injection of DNP_8-MGG. The epitope density of DNP-MGG was shown to be the determining factor in establishing the tolerogenicity of the conjugate. The lightly haptenated conjugates ($DNP_{0.5}$-MGG, $DNP_{1.9}$-MGG) were not tolerogenic, moderately haptenated MGG ($DNP_{4.2}$-MGG, DNP_{14}-MGG) was tolerogenic, and the heavily haptenated MGG (DNP_{32}-MGG, DNP_{53}-MGG) was immunogenic (124). They concluded that the suppressive results from the adminstration of DNP_8-MGG were due to the tolerization of B cells, since in adoptive transfer experiments the B cells or bone marrow cells from tolerized mice cooperated poorly, if at all, with Th cells or thymus cells from OA-primed mice (61).

However, not all the conjugates with autologous proteins are tolerogenic. Paley *et al* (62) reported that DNP-coupled to MGG via ϵ-amino group of lysine readily induced tolerance, whereas DNP coupled to MGG via an azo linkage to histidine and tyrosine or via a mustard linkage to carboxyl groups of glutamic or aspartic acids failed to induce tolerance and was, in fact, immunogenic. It was also reported by Vitetta *et al* (58) that DNP-IgG1 and DNP-IgG2a myeloma proteins were extremely effective tolerogens, whereas DNP-IgG3 was not. Similarly, it was shown in this laboratory by Pan *et al* (89) that whereas DNP_{10}-IgG1 was an effective tolerogen, the other conjugates (i.e. DNP_9-IgM,

DNP_9-IgA, DNP_{10}-IgE, and DNP_{10}-IgG_{2a}) did not affect the anti-DNP IgE response.

Hence, for conjugates of hapten with isologous immunoglobulins, four conditions are critical for the synthesis of tolerogenic conjugates : 1. the hapten density, i.e, the number of haptenic groups coupled per carrier molecule; 2. the retention of the Fc portion , i.e. the immunoglobulin carrier ought to be intact; 3. the mode of linkage of the haptens to the iummunoglobulin carrier. 4. the isotype of the immunoglobulin carrier.

An early study by Battisto & Bloom (63) indicated that haptenated autologous spleen cells, erythrocytes and peritoneal exudate cells were all capable of functioning as tolerogenic carriers for the trinitrophenyl (TNP) group. This discovery was confirmed by several other authors, with respect to both humoral and delayed type hypersensitivity (64, 75). Although the fact that haptenated autologous cells cause immunological unresponsiveness is widely accepted, the mechanism varies from one system to another, and is not always well understood. Fidler (64) claimed that clonal deletion was involved in the tolerance induced by trinitrobenzenesulfonic acid. In other systems, clonal deletion and/or Ts cells were considered to be responsible for the immunologic unresponsiveness (75,76).

In the past several years, Dorf *et al* (68-74) extensively investigated the mechanism of immune unresponsiveness induced by NP-syngeneic (NP = 4-hydroxy-3-nitrophenyl acetyl) spleen cells. In this model system, both cell-mediated and humoral immunity were analyzed. Based on their findings, they proposed that at least three

distinct subsets of Ts cells are involved in the immune suppression, which were designated as Ts1, Ts2, and Ts3, and these cells formed a linear scheme of cellular interactions. Thus, Ts1 cells were shown to be Lyt 1+2- and capable of binding the NP determinants. They function only during the afferent or induction phase of the immune response. Ts1 cells and their factors (TsF1) stimulate a subpopulation of T cells to become Ts2 cells that are idiotype-specific rather than antigen-specific and bear Lyt-2 and I-J determinants. Whereas Ts1 and their factors have no genetic restriction, i.e., they can induce Ts2 cells in any other strain of mice; Ts2 cells are genetically restricted with repect to genes in both Igh and the I-J complex. Hence, Ts2 cells and their factors are effective only when they are adoptively transferred into recipients that are homologous at both the H-2 (I-J) and IgH gene complexes. Although Ts2 are manifested in the effector phase of the immune response, they may not be the final effectors of suppression, this function being performed by the third population of Ts cells, namely Ts3 cells which appear to be activated by Ts2 cells or their factors. Ts3 cells bear I-J and Lyt-2 determinants, are antigen-specific, H-2 and Igh-restricted, and cyclophosphamide-sensitive. In the azobenzene arsonate system, similar conclusions were reached (75).

A number of workers have shown that administration by mouth or intravenous or intraperitoneal injections of chemically reactive haptens led to specific tolerance of both the humoral and cellular responses (76,77,78). In general, the unresponsiveness is hapten-specific, long-lasting and dose-dependent. Suppressor T cells

have been also implicated. Tolerance induction in this manner is probably comparable to tolerance induction with hapten-conjugated autologous or syngeneic cells (64).

Katz *et al* (24) systematically investigated the tolerance induced by DNP-DGL and showed that: 1) The tolerance induced in both unprimed animals or in animals previously immunized with DNP-KLH; was highly hapten-specific, the anti-carrier response not being affected. Moreover, not only the anti-DNP IgE, but also the anti-DNP IgG and IgM responses were suppressed ; 2) the anergic state of tolerized spleen cells was relatively long-lasting, in the animals in which they had been induced as well as in animals which received cells by adoptive-transfer ; 3) the tolerant state was accompanied by a significant diminution of DNP-specific binding cells and it was shown that the high affinity anti-DNP antibody response was preferentially suppressed; 4) it was relatively difficult to reverse the B cell tolerance, induced by DNP-DGL , as shown by the facts that (a) the tolerant state of the spleen cells was not reversed by either passive incubation of cells *in vitro* or by adoptive transfers into sublethally iradiated recipients; (b) a potent nonspecific T cell stimulus, such as that provided by the allogeneic effect, could not reverse the tolerant state of spleen cells, and (c) the enzymatic treatment of cells with trypsin did not reverse the tolerance induced by DNP-DGL; 5) No Ts cells were demonstrated in the tolerance induced by DNP-DGL, i.e., the spleen cells from DNP-DGL treated donors failed to suppress the co-transferred DNP-primed cells from a second donor in adoptive transfer experiments. The results of this experiment indicated that

the response of DNP-KLH primed spleen cells from non-tolerant donors was unaffected by a concomitant transfer of a tolerant cell population which was even in great excess. This observation argues strongly, not only against the possible involvement of Ts cells, but also against the possible " carry-over " effect, i.e., that small amount of the tolerogenic DNP-DGL may have been transferred to the recipients along with the cells from animals which had been suppressed by DNP-DGL. It is also worth noting that Liu *et al* (123) demonstrated that administration of their highly substituted conjugate of TMA with DGL of molecular weight of 63,700 (TM_{36}-DGL) completely suppressed the anti-TM IgE response and reduced substantially also the levels of anti-TM antibodies belonging to other classes.

Other polymeric nonimmunogenic molecules, such as levan (79,80), dextran (80,81), carboxymethyl cellulose (82) can also serve as effective carriers for induction of hapten-specifc unresponsiveness, which was shown to be due to the inactivation of B cells. The participation of Ts cells were not demonstrated in these systems.

Dintzis *et al* (83,84,85,86) tested the T cell independent immunological responses of naive mice to a series of size-fractionated DNP-polyacrylamide molecules and found that molecules with the haptenic epitopes below a certain critical number (about 20) were capable of inhibiting the anti-DNP responses. Increasing hapten density in a molecule at or above threshold size (MW = 100,000) and hapten number (ca. 20) increases its immunogenicity. However, increasing hapten density below threshold size increases its tolerogenicity.

Even though polyethylene glycol (PEG) or methoxyl-PEG (mPEG)

conjugates of protein antigens proved to be tolerogenic, DNP-mPEG was previously shown in this laboratory tto be devoid of tolerogeniity (67). Hence, another analogue to PEG, polyvinyl alcohol (PVA) was employed to induce anti-hapten tolerance.

Polyvinyl alcohol is a non-immunogenic, hydrophilic polymer consisting of the monomeric units of (CH_2CH-OH) and hence it is possible to couple onto it varying numbers of haptenic groups by reaction with the hydroxyl groups of the CH_2-CH backbone. Lee and Sehon (67,90,91,92,93) synthesized benzylpenicilloy-PVA (BPO-PVA) and DNP-PVA for inducing specific suppression to BPO and DNP. These conjugates were shown to result in an immunologically hapten-specific suppression not only with respect to IgE antibodies, but also to other classes of immunoglobulins. Moreover, these tolerogenic conjugates were capable of suppressing the induction of a *de novo* anti-hapten antibody response, as well as an ongoing antibody response. It is interesting to note that even conjugates with an average valence of less than 1, i.e., $DNP_{0.5}$-PVA_{10}, were still capable of inducing hapten-specific suppression (90). Considering the high affinity of IgE antibodies for mast cells, it may be visualized that the level of circulating IgE antibodies did not reflect the true state of sensitivity of these animals, but only antibodies in excess of those required to saturate the corresponding mast cell receptors. However, it was shown that the administration of these tolerogenic conjugates resulted in the reduction of histamine release of the animals (94) and conferred protection for the sensitized animals to systemic anaphylactic shock (92).

It was also demonstrated that the unresponsiveness of the spleen cells of mice which had been rendered tolerant by DNP-PVA was maintained even after cell transfer to irradiated syngeneic recipients (91). Thus, in adoptive transfer experiments, the interaction of DNP-specific B cells with OA-specific T cells of primed mice resulted, on challenge of the cell recipients with a sensitizing dose of DNP-OA, in a marked anti-DNP IgE response. However, the B cells of mice which had been treated with DNP-PVA cooperated poorly with OA-specific T cells and produced only low levels of anti-DNP IgE antibody. Furthermore, the B cells from tolerized mice did not affect the T-B cell cooperation in the production of anti-DNP IgE response. However, spleen cells of mice treated with $DNP_{2.4}$-PVA_{10} 7 days before suppressed (a) the ability of normal nonirradiated syngeneic mice to mount an anti-DNP IgE response on immunization with DNP_3-OA in presence of $Al(OH)_3$, (b) the capacity of immune spleen cells to mount a secondary anti-DNP IgE response in irradiated syngeneic recipients. It should be noted, that in the above experiments, the anti-carrier (i.e., anti-OA) response was not affected. These results were interpreted as indicating that treatment of mice with DNP-PVA resulted in (a) hapten-specific tolerance of B cells and (b) induction of hapten specific Ts cells, whose suppressive capacity was shown to be eliminated by treatment with cyclophosphamide (91,93). However, the relative importance of these two factors in the induction of tolerance was not evaluated.

7. Allergic syndromes induced by trimellitic anhydride (TMA)

Trimellitic anhydride , a highly reactive chemical due to its anhydride group, is widely used in the plastics industry as a plasticizer, curing agent and in surface coatings. Workers exposed to fumes or dust of TMA have been found to develop a spectrum of respiratory syndromes, which include asthma and rhinitis of the immediate type, late respiratory systemic syndrome (LRSS) or 'TMA-flu', airway irritation and most severe of all, pulmonary edema and hemorrhage with anemia (95,96,98,99). These TMA-induced syndromes are considered to be related to the high chemical reactivity of this compound, which may become readily coupled to human protein, when inhaled to form TM-protein conjugates.

An immunologic basis has been postulated for three of these syndromes, i.e., rhinitis-asthma, LRSS, and pulmonary edema and hemorrhage with anemia. In workers exhibiting the characteristic symptoms of the rhinitis-asthma syndrome, the immunologic nature of this occupational disease was attributed to TM-specifc IgE antibodies which were demonstrated in their sera by the radioallergosorbent test (RAST). In LRSS, the onset of asthma occurred over a latent period of 4-8 hours after exposure and was often associated with systemic symptoms such as fever and arthralgia. Some patients had rheumatoid factor, although no clinical evidence of rheumatoid arthritis was detected. The finding of rheumatoid factor and TM-specific IgG antibodies raised the possibility that immune complexes consisting of TM-specifc antibody and TM-protein may be involved in the pathogenesis

of this syndrome (95,99,100). As stated earlier, workers who inhale a fairly large amount of TMA fumes or dust may develop pulmonary hemorrhage and edema, and they have high levels of anti-TM IgE and IgG antibodies in their sera. It is important to stress that Patterson *et al* (101,102) have shown that the anti-TM human antibodies produced by these workers were directly against new determinants on modified protein molecules (e.g., human serum albumin) with the TM groups forming only part of the new determinants .

More recently Akiyama *et al* (103), as an extension of hypothesis, proposed a new mechanism of immediate hypersensitivity induced by hyperreactive chemicals, such as TMA. They suggested that TMA on inhalation, in addition to inducing the production of anti-TM antibodies, may react with various autologous respiratory tract proteins including IgE and Fcϵ receptors on the mast cells and basophils. With continuing exposure to TMA, the anti-TM antibodies would react with haptenated IgE molecules bound to Fcϵ receptors or haptenated receptors on the mast cell and basophil surface and trigger histamine release. In principle, this mechanism may be operative in the absence of serum anti-TM IgE antibodies, as long as other classes of anti-TM antibodies are present . The validity of this hypothesis still needs to be tested.

It was recently demonstrated in this laboratory that conjugates of haptenic groups, such as BPO or DNP or NP groups, with PVA could specifically prevent the induction of the respective anti-hapten antibody responses as well as abrogate established response to the hapten in question. In view of this observation, the possibility was

envisaged that conjugates of TMA with PVA may also possess the potential of abrogating anti-TM antibodies and of being used for therapeutic purposes in man. Hence, the present study was undertaken to test the hypothesis with the aid of a mouse model, that TM-PVA conjugates are immunosuppressive with respect to the TM residue and to investigate the mechanism(s) underlying the unresponsiveness.

MATERIALS AND METHODS

1. Reagents:

Bovine serum albumin (BSA) (Fraction V) and ovalbumin (5 x recrystallized) were purchased from Nutritional Biochemical Corp., Cleveland, OH, TMA from Eastman Kodak Co., Rochester, NY, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide, HCl (EDCI) from Calbiochem-Behring, Corp. La Jolla, CA, and two PVA batches with average molecular weights of 3,000 and 14,000 daltons referred to , repectively, as PVA_3 and PVA_{14}, from Aldrich Chemical Co.,Inc., Milwaukee, WI. Pyridine and N,N-dimethyl formamide (DMF) were purchased from Fisher Scientific, Fair Lawn, New Jersey. The batches of TM-caproic acid was prepared by Professor J. Charlton of the Department of Chemistry , the University of Manitoba. Goat anti-mouse IgE (GAME) and sheep anti-mouse Fab (SAFab) antibodies, purified by affinity chromatography, were kindly supplied by Dr. K. Kelly, formerly of this Laboratory. They were used in appropriate RASTs for determination of specific anti-TM IgE and total antibodies. The extract of Asc was prepared by Ms Valerie Cripps, formerly of this Laboratory.

2. Preparation of TM-protein conjugates

For coupling to BSA, OA, Asc or RNase the method of Zeiss *et al* was used (95). Briefly, proteins were dissolved in 9% sodium bicarbonate solution at a concentration of 10 mg/ml. Different amounts

of TMA powder, dissolved in dioxane, were added dropwise to the protein solution with constant stirring. The mixture was kept in an ice bath with stirring for one hour. Thereafter, the solution was first dialyzed against 9% $NaHCO_3$ overnight and then against phosphate buffered saline (PBS) (0.01 M phosphate buffer, pH 7.2, 0.15 M sodium chloride) until the optical density (OD) of the dialysate at 240 nm was close to zero.

The hapten valence, or epitope density, defined as the number of trimellityl groups coupled per carrier molecule in the TM-protein conjugate was determined spectrophotometrically (95), assuming that the molar extinction coefficient of TM was not affected by conjugation.

As may be seen from the absorption spectrum of Na_3TM in Fig 1, the absorbance was highest near 200 nm, dropped quickly at 250 nm, with a specific absorption shoulder in the vicinity of 240 nm and a peak near 280 nm. The extinction coefficient of Na_3TM at 240 nm is 11,400 (95). For TM- protein conjugates there was a small contribution of the TM group to the absorption of protein at 280 nm. However, proteins absorb also significantly at 240 nm. For the calculation of the epitope density, solutions of BSA, OA and RNase and Na_3TM were prepared, and the absorbance was read for each solution both at 240 nm and 280 nm. The ratio $OD_{240}:OD_{280}$ for each protein or TMA was thus established. The ratio $OD_{240}:OD_{280}$ for Na_3TM was determined to be 0.125. The calculations were performed as follows:

Assuming that T = OD_{240} due to TM

P = OD_{280} due to protein (BSA,OA or RNase)

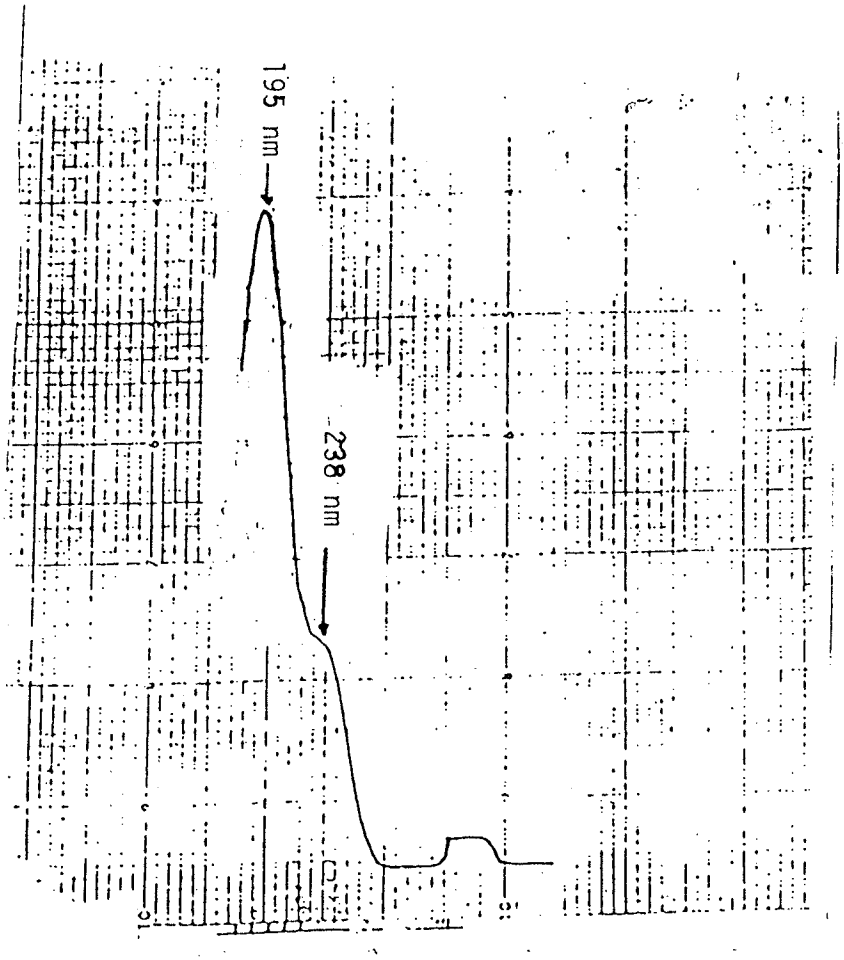


Fig 1. O.D. profile of Na_3TM

E = observed OD_{280} of TM-protein

F = observed OD_{240} of TM-protein

then at 280 nm

E = P + 0.125 T

F = OD_{240}:OD_{280} (of proteins) P + T

T and P can thus be calculated from the above equations.

3. The preparation of conjugates of TM with PVA

Two procedures were developed for coupling TM groups to PVA, one involving a linker molecule of hexanediamine and the other the direct reaction of TMA with PVA.

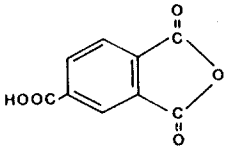
(i) TM-NH-PVA For the first mentioned method, the amino derivatives of PVA were prepared by reacting 1,6-hexanediamine with different batches of PVA, which had been activated by cyanogen bromide as previously described (90). The corresponding conjugates, of differing epitope density, designated as TM_n-NH-PVA where $_n$ represents the average number of TM groups attached per PVA chain., (i.e., $TM_{0.5}$-NH-PVA_3 , $TM_{0.9}$-NH-PVA_3 , and $TM_{1.7}$-NH-PVA_{14}), were prepared by the addition of 0.5 gm of TMA dissolved in dioxane to 10 ml of an aqueous solution of 100 mg of aminated PVA. The reaction mixture was stirred for three hours or overnight and then dialyzed against multiple changes of distilled water until the OD_{240} of the dialysate was close to zero. The conjugates were then lyophilized and the epitope density determined spectrophotometrically.

(ii) TM-PVA conjugates Since the epitope density of the TM-NH-PVA conjugates was rather low, the following procedure was employed for the preparation of conjugates of higher epitope densities. For this purpose 2 gm of PVA_3 or PVA_{14} were dissolved in 100 ml of pyridine or dimethyl formamide under reflux. After the solution was cooled to room temperature, 1 gm of TMA powder was added to the solution and the mixture was refluxed for half an hour. The conjugates were precipitated with pentane and then washed 3 times with benzene. The precipitate was redissolved in 100 ml of distilled water and passed through a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column to remove the residual TMA. The fraction containing the bulk of the polymer was collected, dialyzed extensively against distilled water and lyophilized. From spectrophotometric determinations it was concluded that this method yielded conjugates of higher epitope density and that the two conjugates synthesized by this procedure were represented by the formulae $TM_{1.4}$-PVA_3 and TM_7-PVA_{14}.

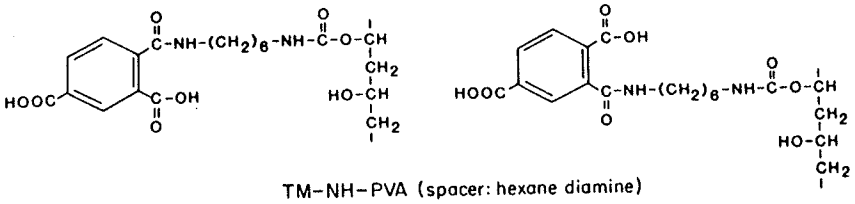
Clearly, because of the asymmetry of TMA, it is plausible to suggest that the two sterically isomeric products illustrated in Fig 2 would be formed in the reaction with PVA or its aminated derivative. Similar substitution products would be expected to be formed during the reaction of TMA with proteins which would involve primarily the free ϵ -NH_2 groups of the lysine residues.

4. Animals and immunization

Female 6-12 week old (C57BL/6 x DBA/2) F1 mice referred to hereafter as BDF1 , male C57BL/6 mice, and BALB/C female mice, and



Trimellitic anhydride (TMA)



TM-NH-PVA (spacer: hexane diamine)

TM-PVA

Figure 2. Trimellitic anhydride and its derivatives, TM-NH-PVA and TM-PVA. (Each type of derivative may be represented by two sterioisomers which are produced during the synthesis because of the asymmetrical structure of TMA).

random bred Long-Evan hooded rats were obstained from the Animal Care Facility of the University of Manitoba.

The mice were injected intraperitoneally with a sensitizing dose of 1 μg of TM_3-OA or TM_9-OA suspended in 1 mg of freshly prepared $Al(OH)_3$ gel in 0.5 ml of PBS (92). In some experiments a second sensitizing dose was given about 4 weeks later (exact protocols are indicated under RESULTS). Each test group consisted of 4 mice; the sera of mice within each group were pooled for the determination of anti-TM and anti-OA or other antibodies.

In all the experiments, each group of mice consisted of 4 unless specifically mentioned.

5. Passive cutaneous anaphylaxis (PCA)

The biological activity of IgE antibodies was determined by PCA in hooded rats. The pooled mouse sera were first serially diluted in saline and 50 μl of each serum dilution was injected intradermally into the dorsal skin of the rats, which were challenged 24 hours later by intravenous injection of 1 mg of TM_{15}-BSA or 1 mg OA in 1 ml 0.5% Evans' blue dye. The PCA titre was taken as the reciprocal of the highest dilution of each serum resulting in a reaction of 5 mm in diameter. The PCA titres were reported as geometric means of two determinations in two different rats (92). For a given serum, the difference of PCA titres tested on duplicate rats was never more than 2-fold. A difference of 4-fold in PCA titres among the pooled sera was considered significant.

For PCA determination of rat IgE, 100 μl of each serum dilution was injected intradermally into the back of hooded rats. After 48 hours, 1 mg of the corresponding antigen was injected i.v. with Evans' blue and the rest of the procedure was the same as done in mice.

6. Determination of TM- and OA-specific antibodies of the IgE and other classes by RASTs

Ovalbumin and BSA were covalently attached to paper discs by a slight modification of methods previously described (104). The TM-BSA discs were prepared by coupling TMA to BSA discs. For this purpose, 5 gm of finely powdered TMA was added with stirring over one hour to a suspension of 5 gm of BSA coated discs in 125 ml of 9% $NaHCO_3$ solution, kept in an ice bath. Gentle shaking was continued overnight at 4°C and the discs were then washed with 9% $NaHCO_3$ solution.

The GAME and SAFab antibodies were radio-iodinated with ^{125}I (Amersham Corp., UK) by the chloramine T method as previously described (105). One hundred μg of proteins were labelled with 1 mCi of ^{125}I and the free iodide was removed by gel filtration through Sephadex G-25 column .

The assays for anti-TM and anti-OA IgE antibodies were performed essentially as described previously (105) utilizing the labelled GAME. The reciprocal of the serum dilution which yielded 3% bound radioactivity was arbitrarily considered as the RAST titre. The method for quantitation of total anti-TM and anti-OA antibodies was identical to the procedure described above except that ^{125}I-SAFab was substituted

for ^{125}I-GAME and the assay buffer contained 10 % normal sheep serum.

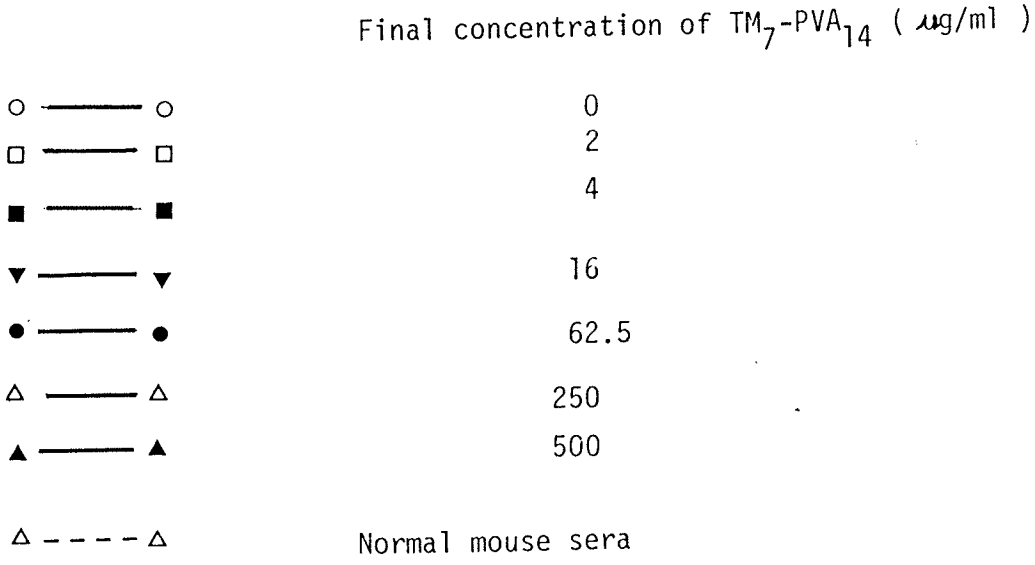
7. RAST inhibition assay

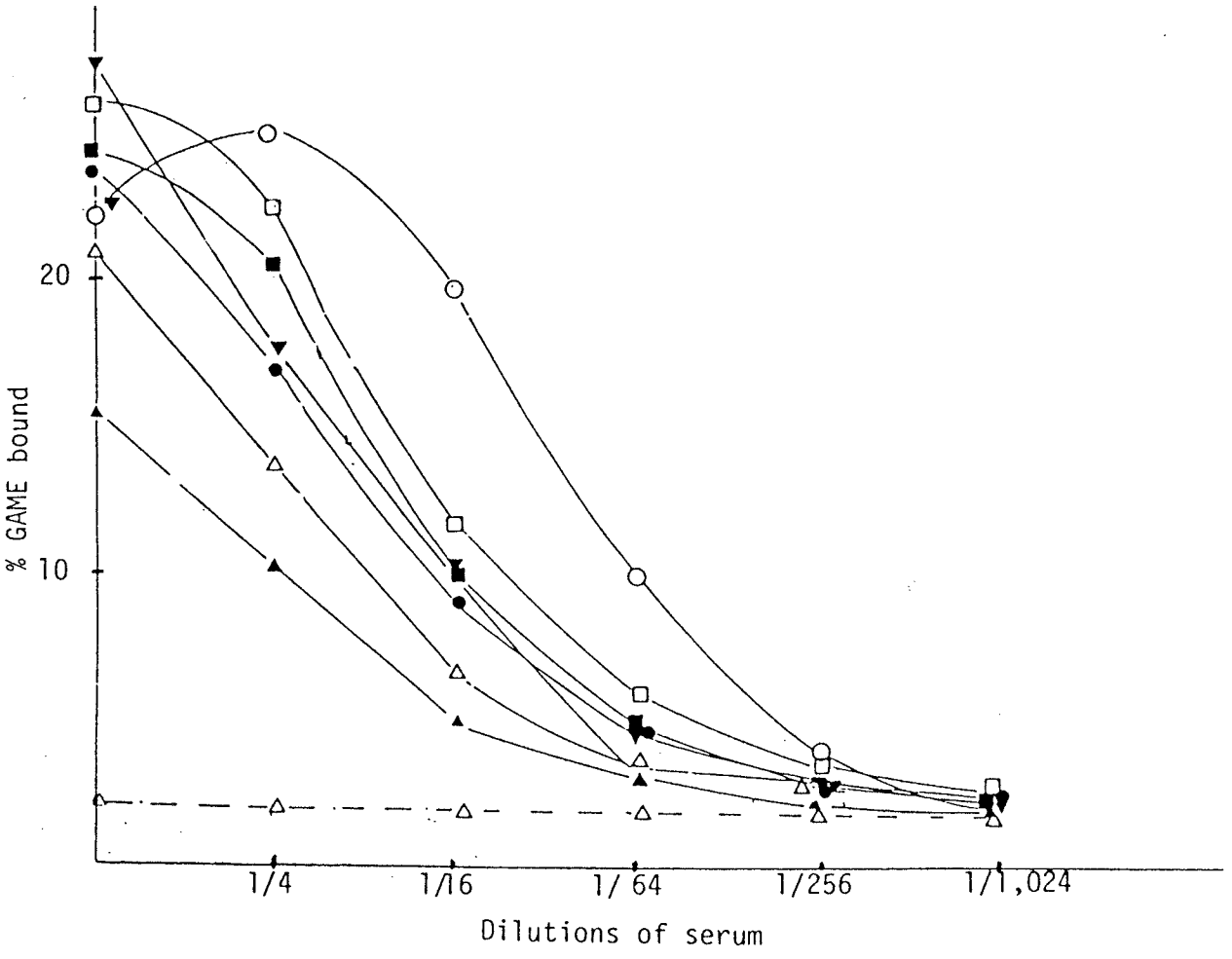
For the determination of serum levels of TM_7-PVA_{14}, a pool of murine sera with a PCA titre of 1,300 was used as a standard serum in the RAST. For the construction of a reference curve for the RAST inhibition assays, increasing doses of TM_7-PVA_{14} were incubated with different dilutions of the standard serum at room temperature for 3 hours; the mixtures were then incubated with either TM-BSA coated discs or OA discs for the RAST IgE assays. This RAST inhibition assay was rather specific, since the anti-OA curves were not influenced by the incubation of the sera with TM_7-PVA_{14}. However, the incubation of the sera with TM_7-PVA_{14} significantly inhibited the binding of anti-TM IgE antibodies with the TM-BSA coated discs (Fig 3) and, as illustrated that with the decreasing amounts of TM_7-PVA_{14} during incubation the RAST binding curves approached the controls (i.e., without TM_7-PVA_{14} during incubation). This RAST inhibition assay was sensitive enough to detect as little as 2 μg/ml of TM_7-PVA_{14} .

To establish a standard reference line for these assays, a 1:4 dilution of a "standard serum" was used, because at this dilution there was good separation among all the standard binding curves in the RAST inhibition assays. By interpolation, from the standard reference lines (Fig 4), it was possible to determine the amounts of free TM_7-PVA_{14} in the serum as a function of their RAST inhibition capacity.

Fig 3. RAST inhibition curves by TM_7-PVA_{14}

The standard serum at a series of dilutions was incubated with different concentration of TM_7-PVA_{14} in PBS for 3 hours at room temperature. The mixture was then tested for anti-TM IgE antibodies by the standard RAST assays.




20
10
% GAME bound
1/4
1/16
1/64
1/256
1/1,024
Dilutions of serum

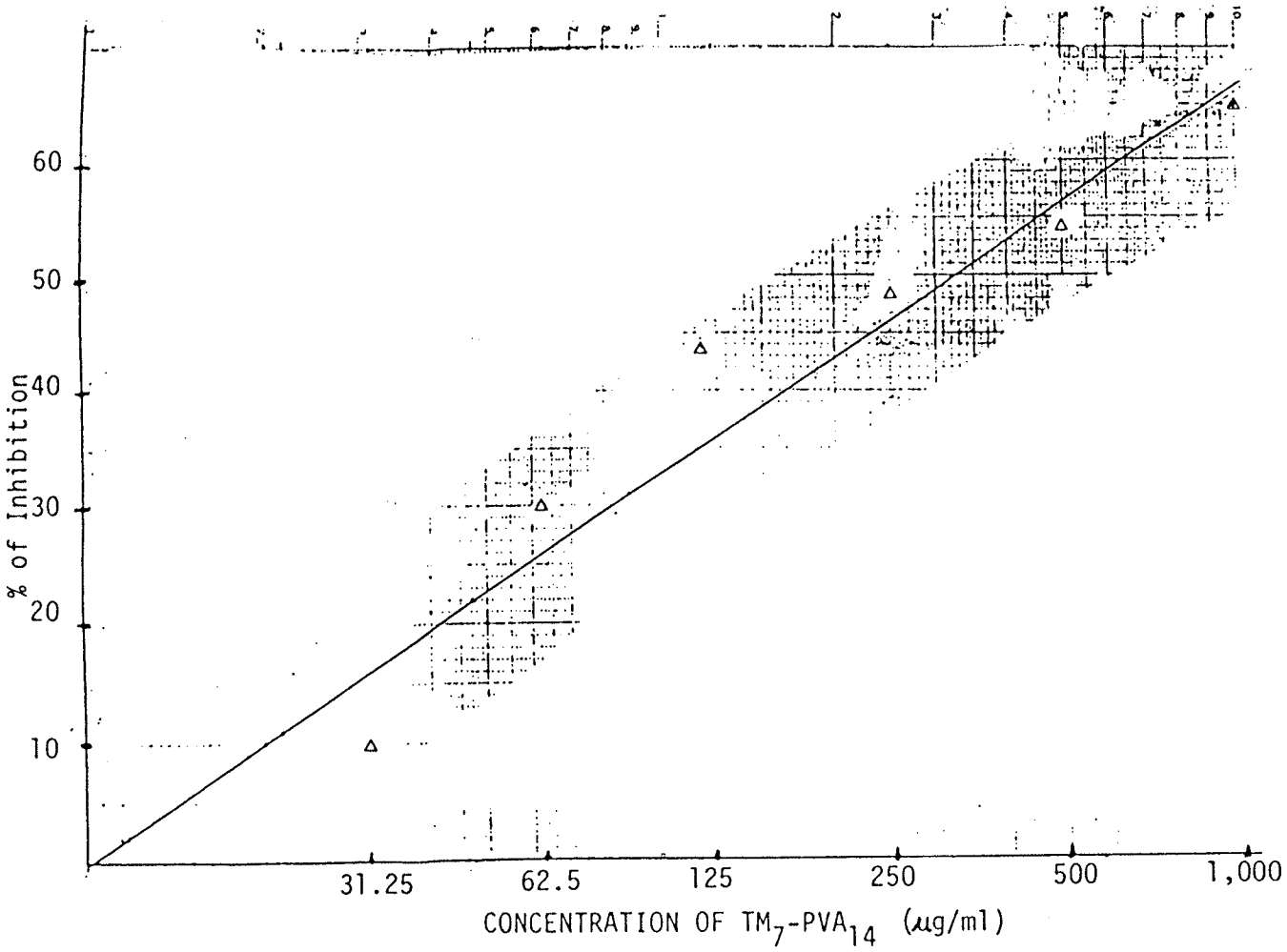


Fig 4. Standard reference line for determining TM_7-PVA_{14} concentrations based on inhibition of RAST.

A standard serum of 1:4 dilutions was incubated with different concentrations of TM_7-PVA_{14}. After incubation at room temperature for 3 hours, the mixtures were used for RAST assays. The percentage of inhibition (relative to the titre due to serum incubated with only PBS) is represented by the ordinate and the concentration of TM_7-PVA_{14} used in the inhibition assay is shown on the abscissa.

8. Preparation of radioactive $N^{125}IP\text{-}PVA_{14}$

Radiolabelled $N^{125}IP\text{-}PVA_{14}$ was prepared from $NP_4\text{-}PVA_{14}$ by the chloramine T method (the sample of $NP_4\text{-}PVA_{14}$ was prepared by Mr Stephan Krueger of this Laboratory. For this purpose, a solution containing 100 μg of $NP_4\text{-}PVA_{14}$ in 100 μl was mixed with 25 μl of 0.5M phosphate buffer and 0.5 μl of $Na^{125}I$ (0.5 mCi) and 25 μl of chloramine T (4 mg/ml), and the mixture was vortexed for 45 seconds. To stop the reaction, 100 μl of $Na_2S_2O_5$ and 200 μl of KI (10 mg/ml) were added and the mixture was fractionated by two passages through a Sephadex G-25 (1.5 cm^2 x 30 cm); the first emerging fraction contained the radio-labelled conjugate.

9. Preparation of lymphoid cells supensions (106)

The spleens, lymph nodes and thymuses were collected from mice. Single cell suspensions were prepared in Eagle's minimum medium (MEM) buffered with 20 mM Hepes, and were washed twice by gentle centrifugation at 4°C, and then suspended in cold medium.

To remove the red cells and dead cells, the single cell suspensions were carefully layered on Ficoll-Metrizoate and centrifuged at 350 g for 20 minutes. The white cells at the interface were collected, washed and used as lymphoid cells.

10. Enrichment of T cells by nylon wool column

The procedure was basically the same as previously reported

(106). Briefly , nylon wool (Fenwall Laboratories, LP-1 Leuko-Pak Leukocyte Filters) was boiled, teased apart, packed into 10 ml disposable syringes, and autoclaved for further use. To enrich the T cells, the lymphocyte suspension in 5% FCS-MEM-Hepes ($1x10^6$ cells/syringe) was incubated at 37°C for 1 hour in the syringe which had been washed with MEM-Hepes. The non-adherent cells were considered to be enriched T cells.

11. Irradiation of mice

The mice were exposed to different doses of whole body irradiation from a ^{60}Co source (Theratron F Cobalt Unit). In adoptive transfer experiments, the recipient mice were exposed 18-24 hours before transfusion of cell suspensions (106).

12. Bone marrow cell transfusion (106)

After irradiation with a hyperlethal dose (1,000 rads) , the mice were i.v. transfused with bone marrow cells from normal syngeneic animals. The donor mice were killed by cervical dislocation , their muscles and epiphyses were removed from their femura and tibiae . The bone marrow cells were washed out with cold MEM by a 20 gauge needle and passed through a stainlesss steel mesh to obtain single cell suspensions.

13. Adoptive cell transfer (106)

In the adoptive cell transfer experiments, spleen cells were removed from the donor mice at a specific time after sensitization

and/or treatment. Single cell suspensions were prepared in MEM buffered with 20 mM Hepes, washed and transferred i.v. into syngeneic recipient mice. The recipients were then injected i.p. with antigen, after the time interval specified in the experimental data section.

14. Cytotoxicity with anti-Thy 1.2 (F7D5) plus complement (107)

Anti-Thy 1.2 (F7D5) monoclonal antibody of the IgM class was purchased from Shaw's Farm, Blackhorn, Bicester, Oxon, U.K. It was dissolved in 5% FCS-MEM at dilutions from 1/500 to 1/20,000. Lymphocytes were incubated at a concentration of $1x10^7$ cells/ml at room temperature for 30 minutes with different concentrations of anti-Thy 1.2 antibodies. The cell suspension was then centrifuged and the pellet was resuspended in a 1:10 low toxicity rabbit complement (CL 3051, Cederlane Lab Ltd, Hornby, Ontario, Canada) at 37°C for 40 minutes. The cells were washed, centrifuged and collected for further experiments.

The appropriate concentration of anti-Thy 1.2 antibody preparation was determined by cytotoxity tests with normal thymocytes, in which at least 90% of thymocytes and about 30% of spleen cells are expected to be killed.

The rabbit complement was absorbed with agarose before use.

15. The preparation of anti-ϵ developing serum

This is a cooperative work among several people in this Department. Rabbits were immunized with monoclonal IgE produced by the hybridoma #1588 (anti-NP, $\epsilon_2\lambda_2$, $\epsilon_2\kappa_2$) generated from the parent strain of CB6F1

in the Hybridoma Unit of this Department. The rabbit serum was first absorbed with HOPC-1 (γ_{2a} λ_2) Sepharose 4B , and then with normal mouse IgG-Sepharose 4B . The effluent portion was subsequently passed through a IgE 2682-Sepharose 4B [anti-DNP, (BALB/C x A/J)F1]. The adherent fraction was eluted by a solution of 4 M guanidine-HCl in 0.25 M acetic acid (pH 4.0) and neutralized with pH 8.0, 1 M Tris-HCl buffer; and then dialyzed against PBS prior to concentration. The product was further purified by affinity chromatography through normal mouse IgG-Sepharose 4B and HOPC-1 Sepharose 4B immunosorbents, and the effluent was collected and aliquoted. Before and after each step, the removal of the unwanted antibodies was monitored by immunodiffusion. A pilot absorption experiment was always done to determine the amount of the immunosorbent needed.

16. Plaque Forming Cell (PFC) assays

All the PFC assays were performed according to Cunningham's method (108). For the enumeration of anti-TM and anti-DNP IgE-PFCs, 0.4 ml packed sheep red blood cells (SRBC) were mixed with 50 mg of TM_{25}-BSA or DNP_{28}-BSA in 6 ml saline, and a solution of 20 mg of EDCI in 0.4 ml saline was added dropwise. After gently rotating the mixture at room temperature for 45 minutes, it was washed with PBS three times. The developing rabbit anti-mouse serum was used at a dilution of 1:32, which had been shown to give a maximum number of IgE-PFC in a previous calibration experiment. For the subtraction of the direct IgM plaques, which were produced along with the IgE-PFCs, the former were determined in a separate chamber under identical conditions except for the absence

of the anti-ϵ serum. Every sample was tested in duplicate, and the mean value was recorded. The specificity of anti-TM and anti-DNP plaques had been confirmed by the inhibition of the respective PFCs by incorporation of either TM_{25}-BSA or DNP-ϵ-aminolysine into the cell suspension. To establish if TM_7-PVA_{14} had any nonspecific effect on the production of PFCs, SRBC IgM-PFC determined 3 days after immunization of mice with 4×10^8 SRBC served as an additional control.

RESULTS

I. Suppression of anti-TM IgE and non-IgE antibodies by conjugates of PVA

In recent years, an increasing number of low molecular weight industrial chemicals have been shown to be responsible for occupational asthma and other respiratory diseases (109,110,112). The underlying mechanisms for some conditions caused by chemicals, such as TDI, have been shown to involve β-adrenergic blocking activity in vitro (111). However, for other chemicals, such as TMA and plicatic acid (113), as well as for TDI (114), the participation of immune mechanisms involving antibodies of the IgE and other classes was shown to be implicated. These compounds have a common characteristic,i.e., they are extremely reactive with amino and hydroxyl groups. Hence, it may be visualized that on inhalation, they may react with soluble or cell membrane proteins of the respiratory tract and thus assume their immunogenic properties in the form of the corresponding hapten-protein conjugates.

In this phase of the study, TM-PVA conjugates were synthesized and tested for the suppression of anti-TM IgE antibody and other classes.

1. Abrogation of the induction of anti-TM antibodies

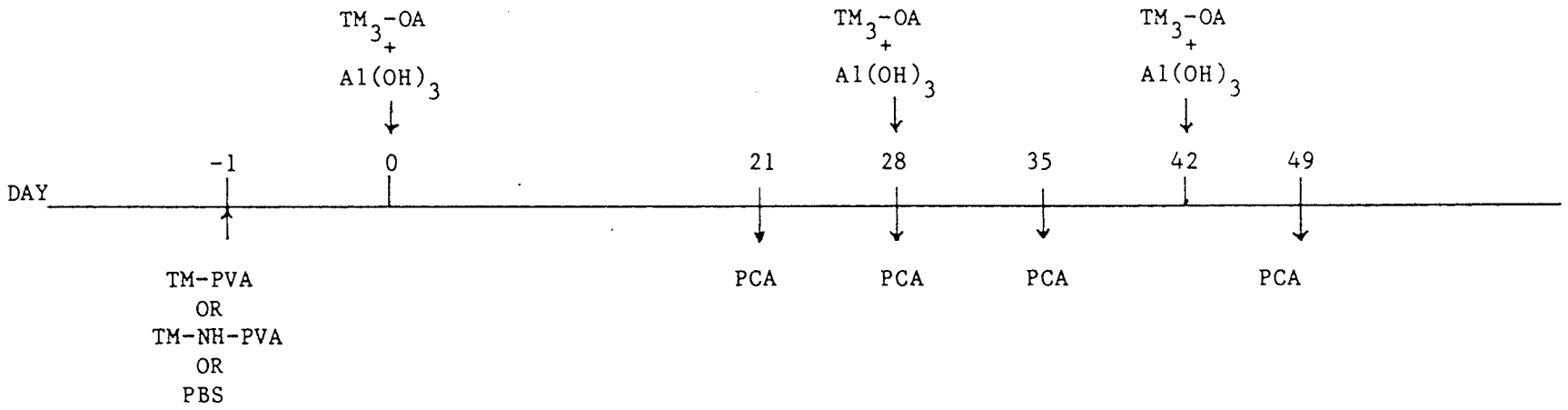
The mice in the test groups received an i.p. injection of 1 mg of $TM_{0.9}$-NH-PVA_3, or $TM_{1.4}$-PVA_3 or $TM_{1.7}$-NH-PVA_{14}, and the control group

received PBS one day before the administration of the first sensitizing dose of TM_3-OA;, animals in all groups received two additional sensitizing doses of TM_3-OA on days 28 and 42. The PCA titres of the sera collected on different days are listed in Table 1, from which it is obvious that induction of the anti-TM IgE response was almost completely suppressed by treatment of the animals with a single dose of 1 mg of any of these conjugates. By contrast, the anti-OA IgE response was not affected by this treatment. It is important to note that all three PVA conjugates, in spite of their low epitope densities, were capable of switching off the anti-TM IgE response for extended periods and that repeated injections of the sensitizing dose of TM_3-OA did not break the established immunological tolerance to the TM group (at least during the period of observation). The results of RASTs performed on sera collected on day 35 confirmed that the formation of anti-TM antibodies belonging to IgE, as well as to other immunoglobulin classes had been markedly suppressed by pretreatment of the animals with these PVA conjugates (Fig 5 & Table 2). Furthermore, the changes in the slopes of RAST binding curves (Fig. 5) probably reflect that the anti-TM IgE antibodies of high affinities were suppressed.

2. Suppression of the anti-TM response in presensitized mice

As illustrated in the protocol of Table 3, all the mice in the test and control groups received two sensitizing doses of TM_3-OA on days 0 and 28. On day 26, each of the mice in the four test groups

TABLE 1. SUPPRESSION OF INDUCTION OF THE ANTI-TM IgE RESPONSES.



TREATMENT	PCA TITRES									
	PRIMARY				SECONDARY				TERTIARY	
	DAY 21		DAY 28		DAY 35		DAY 42		DAY 49	
	ANTI-TM	ANTI-OA	ANTI-TM	ANTI-OA	ANTI-TM	ANTI-OA	ANTI-TM	ANTI-OA	ANTI-TM	ANTI-OA
PBS	50	1840	50	2690	2920	4320	2690	5750	8580	6170
$TM_{0.9}$-NH-PVA_3	10	1340	10	2690	70	3800	70	2820	80	5750
$TM_{1.4}$-PVA_3	10	2920	10	3160	10	5820	10	6170	40	11980
$TM_{1.7}$-NH-PVA_{14}	10	2690	10	2780	70	6170	80	6720	300	9190

All mice received a sensitizing dose of TM_3-OA on each of days 0, 28 and 42. One day before primary immunizatio each of the test mice received 1 mg of the indicated PVA conjugate in 0.5 ml PBS; the control group received 0.5 ml PB The PCA titres were determined on days 21, 28, 35, 42 and 49 (the mice were bled on day 28 just prior to receiving t booster sensitizing dose of TM3-OA).

TABLE 2. SUPPRESSION OF THE INDUCTION OF THE ANTI-TM RESPONSE: RAST TITRES ON DAY 35 OF THE IMMUNE RESPONSE*

TREATMENT	ANTI-TM IgE AND TOTAL ANTIBODY TITRES DETERMINED BY RASTs	
	IgE	TOTAL ANTIBODY
PBS	232	4×10^4
$TM_{0.9}$-NH-PVA_3	17	1×10^3
$TM_{1.4}$-PVA_3	<4	$<1 \times 10^3$
$TM_{1.7}$-NH-PVA_{14}	5	2×10^3

*For protocol please see Table I.

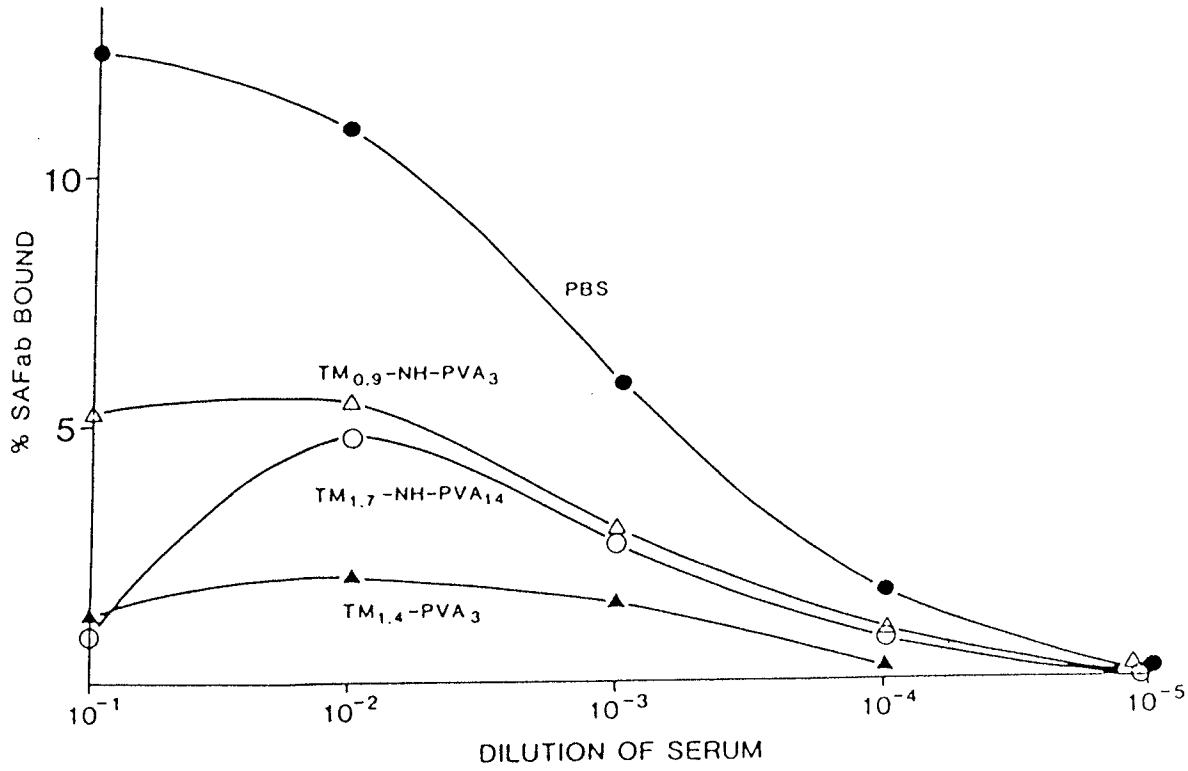
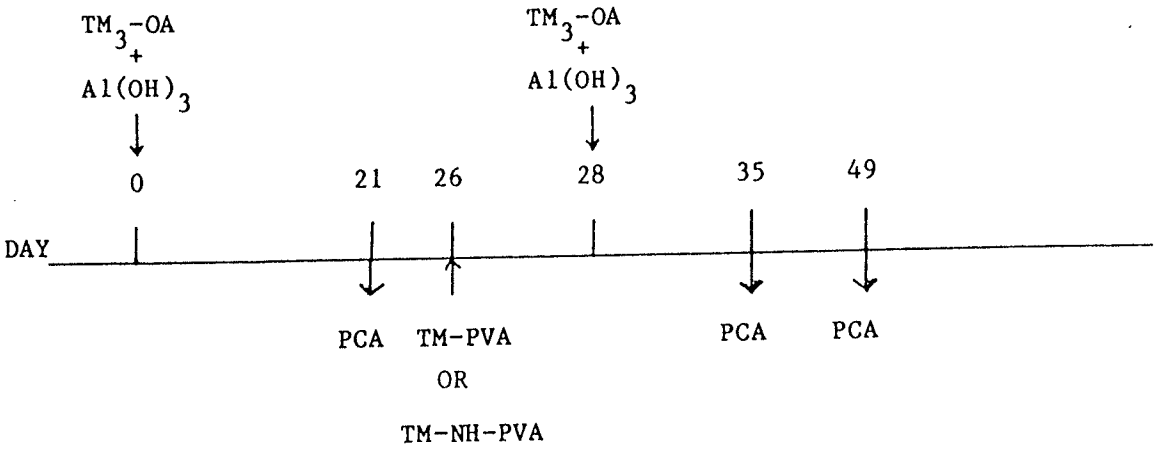


Figure 5: Inhibition of production of total anti-TM antibodies by different tolerogenic conjugates. On days 0 and 28 all mice were immunized with 2 doses of 1 μg TM_3-OA adsorbed on 1 mg $Al(OH)_3$. One day prior to first sensitization, the control group was given PBS (●——●), the test groups were injected i.p. with 1 mg $TM_{0.9}$-NH-PVA_3 (△——△), $TM_{1.7}$-NH-PVA_{14} (o——o) or $TM_{1.4}$-PVA_3 (▲——▲). All the mice were bled on day 35 and their total anti-TM antibodies were measured by RASTs with SAFab.

received an injection of 1 mg of $TM_{0.5}$-NH-PVA_3 , or $TM_{0.9}$-NH-PVA_3, or $TM_{1.4}$-PVA_3 , or TM_7-PVA_{14}, and the control group received only PBS. As is evident from the PCA results listed in this table, all these conjugates markedly suppressed the secondary anti-TM IgE response for extended periods of time. It is remarkable that even the conjugate $TM_{0.5}$-NH-PVA_3 , i.e. a conjugate of very low average epitope density probably with about 50% of the preparation corresponding to a composition of $TM_{1.0}$-NH-PVA_3, was capable of suppressing the anti-TM IgE response by more than 90%. It is also clear from these results that none of the PVA conjugates had any effect on the anti-OA IgE response. From these data it may be concluded that even TM-PVA conjugates with an average density less than 1 were effective in bringing about the suppression of the anti-TM response and that, although the more highly substituted conjugates tended to be more suppressogenic, the relationship between their epitope density and their suppressive effect was not very marked. Although the conjugates were not fractionated into subpopulations containing different epitope densities, these results support the view that these conjugates are tolerogenic even at a very low epitope density, since it is highly unlikely that polyvalent conjugates would have been present in a significant proportion. In Table 4 are listed the results of RASTs performed on sera collected on day 35 which supported the PCA data given in Table 3 and also demonstrated that, in addition to anti-TM IgE antibodies, the anti-TM antibodies belonging to other immunoglobulin classes were also suppressed.

TABLE 3. SUPPRESSION OF ANTI-TM IgE RESPONSE IN SENSITIZED MICE.



TREATMENT	PCA TITRES					
	DAY 21		DAY 35		DAY 49	
	ANTI-TM	ANTI-OA	ANTI-TM	ANTI-OA	ANTI-TM	ANTI-OA
PBS	120	3470	2690	6380	450	5430
$TM_{0.5}$-NH-PVA_3	140	3470	200	6490	20	5750
$TM_{0.9}$-NH-PVA_3	140	3470	20	6720	10	5950
$TM_{1.4}$-PVA_3	70	3320	20	6720	10	6380
TM_7-PVA_{14}	70	3320	10	6170	10	5750

All mice received a sensitizing dose of TM_3-OA on days 0 and 28. The test groups were injected with different preparations of PVA conjugates (1 mg in 0.5 ml PBS) on day 26, while the control group was injected only with PBS. The PCA titres were determined on days 21, 35 and 49.

TABLE 4. SUPPRESSION OF ANTI-TM IgE RESPONSE IN SENSITIZED MICE: RAST TITRES ON DAY 35

TREATMENT	ANTI-TM IgE AND TOTAL ANTIBODY TITRES DETERMINED BY RASTs	
	IgE	TOTAL ANTIBODY
PBS	588	$> 10^6$
$TM_{0.5}$-NH-PVA_3	11	$10^3 - 10^4$
$TM_{0.9}$-NH-PVA_3	8	$10^3 - 10^4$
$TM_{1.4}$-PVA_3	<4	$10^3 - 10^4$
TM_7-PVA_{14}	<4	$< 10^3$

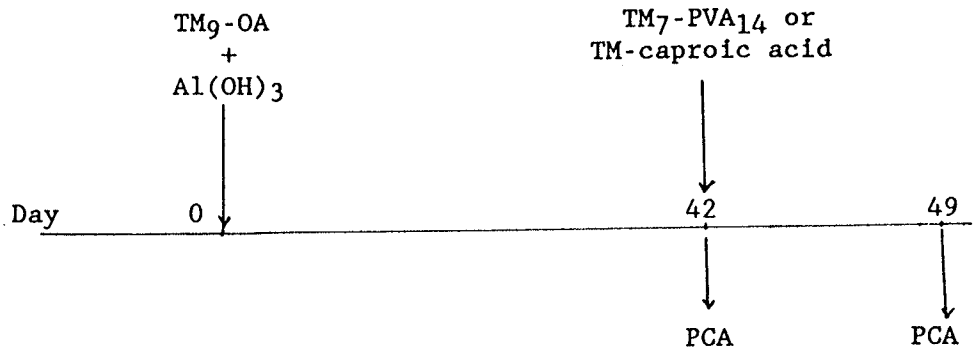
3. The monovalent TM-caproic acid conjugate had no suppressive effect upon anti-TM IgE response

To exclude the possibility that the reduction in anti-TM antibody measured was simply due to the neutralization of the antibodies by the haptenic part of the molecules, TM-caproic acid was synthesized and injected into animals. For this purpose, mice were immunized with a sensitizing dose of TM_9-OA, which elicits a strong immune response. After 42 days, different groups of mice were treated with either TM_7-PVA_{14} or different doses of TM-caproic acid. As is evident from the results in Table 5, 7 days after treatment, mice which had received TM_7-PVA_{14} showed a remarkable reduction of anti-TM IgE antibodies, whereas the sera of mice which had received even a much higher molar excess of TM-caproic acid showed no reduction of anti-TM IgE antibodies at all.

4. The more intense suppressive effect of TM_7-PVA_{14} on the stronger anti-TM response induced by TM_9-OA

The above findings (Table 3) indicated that a single injection of any of the four conjugates of TM prepared with PVA_3 or PVA_{14} reduced dramatically an ongoing anti-TM response induced by TM_3-OA. However, it was noted that although the secondary and tertiary responses to TM were high in mice immunized with TM_3-OA (See Table 1), the primary anti-TM response was considerably lower than that of OA. Hence, in order to test the immunosuppressive potential of TM-PVA conjugates under more stringent conditions, an attempt was made to induce a stronger initial response to TM using the more immunogenic TM_9-OA

Table 5. Comparison of the suppressive effects of TM_7-PVA_{14} and TM-caproic acid



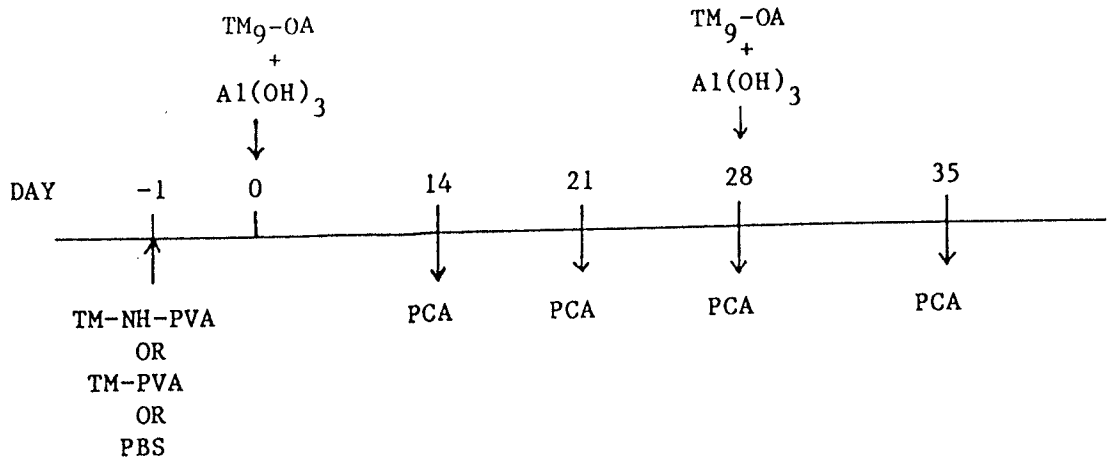
Treatment	Day 42 PCA titres anti-TM	Day 42 PCA titres anti-OA	Day 49 PCA titres anti-TM	Day 49 PCA titres anti-OA
PBS	870	850	830	1,410
TM_7-PVA_{14} (1 mg)	1,280	760	60	1,410
TM-caproic acid 0.01 mg	1,280	1,410	1,410	1,350
0.1 mg	1,350	810	1,280	1,280
1 mg	1,410	810	1,280	1,410

BDF1 mice were immunized on day 0 with a sensitizing dose of TM_9-OA, and on day 42 these mice were bled and injected with 1 mg TM_7-PVA_{14}, or different doses of TM-caproic acid. On day 49, they were bled for PCA determinations.

conjugate. Indeed, the results listed in Table 6 demonstrate that immunization of the control mice with TM_9-OA resulted in a much more intense primary response to TM, reaching PCA titres of the order of 1,000 after a single injection of the sensitizing dose. It is evident that TM-PVA conjugates with an average epitope density of 1.4 as well as TM-NH-PVA_{14} conjugates with average densities between 0.5 and 1.7 were weakly suppressogenic both with regard to the primary and secondary response when administrated before primary immunization . By contrast, the results of the experiment illustrated in Fig 6 demonstrate that injection, even 7 weeks after the initiation of a strong response, of the TM_7-PVA_{14} conjugate, which has similar epitope density per unit PVA length as does the shorter conjugate $TM_{1.4}$-PVA_3 listed in Table 6, was highly effective in reducing the ongoing anti-TM IgE response by more than 96%. Moreover, the results listed in Table 7 indicated convincingly that TM_7-PVA_{14} was also capable of effectively suppressing a strong ongoing anti-TM IgE response even when administered after two sensitizing injections of TM_9-OA and that this hapten-specific suppressogenic effect was dose-dependent, a suppression of the order of 97% being achieved with one i.p. injection of 0.5 mg of TM_7-PVA_{14}. Again, as in previous experiments, the TM_7-PVA_{14} conjugate did not affect the anti-OA IgE response.

Since hapten-PVA conjugates may persist in the host for long time, it is important to confirm that anti-TM antibody production was reduced at the cellular level to exclude the possibility that the reduction of anti-TM antibody was not due to simple antibody neutralization. For this purpose, TM_7-PVA_{14} or PBS was injected into BDF1 mice one day

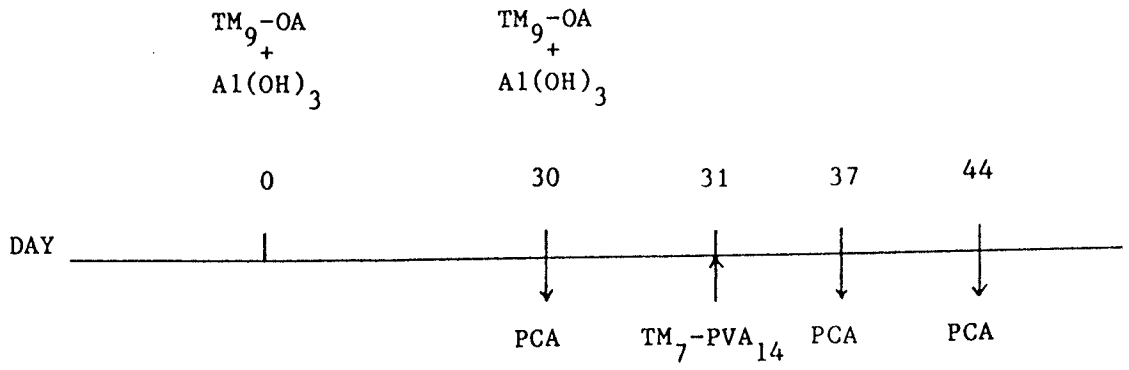
TABLE 6. THE SUPPRESSIVE EFFECT OF DIFFERENT TM-PVA CONJUGATES ON THE ANTI-TM IgE RESPONSE INDUCED BY TM_9-OA



TREATMENT	PCA TITRES							
	DAY 14		DAY 21		DAY 28		DAY 35	
	ANTI-TM	ANTI-OA	ANTI-TM	ANTI-OA	ANTI-TM	ANTI-OA	ANTI-TM	ANTI-OA
PBS	910	640	900	2,560	730	1,900	4,120	5,620
$TM_{0.5}$-NH-PVA_3	790	850	790	1,410	730	2,050	3,040	5,430
$TM_{0.9}$-NH-PVA_3	430	790	240	1,410	120	2,210	2,140	5,590
$TM_{1.4}$-PVA_3	390	790	260	3,020	290	3,790	1,340	10,590
$TM_{1.7}$-NH-PVA_{14}	320	760	550	2,820	290	2,070	2,820	5,820

All mice received a sensitizing dose of TM_9-OA on days 0 and 28. The test groups were injected with 1 mg of PVA conjugates one day before the primary immunization, whereas the control group mice received 0.5 ml PBS. The PCA titres were determined on days 14, 21, 28 and 35.

TABLE 7. SUPPRESSION OF THE ANTI-TM RESPONSE BY DIFFERENT DOSES OF TM_7-PVA_{14}



DOSE OF TM_7-PVA_{14}	PCA TITRES					
	DAY 30		DAY 37		DAY 44	
	ANTI-TM	ANTI-OA	ANTI-TM	ANTI-OA	ANTI-TM	ANTI-OA
PBS	680	1,280	2,920	6,610	2,560	6,610
0.1 mg	640	1,280	990	6,840	780	6,610
0.5 mg	640	1,280	290	6,610	80	5,750
1.0 mg	680	1,280	270	6,380	70	5,750

All mice received a sensitizing dose of TM_9-OA on days 0 and 30. On day 31 the test mice received i.p. injections of different doses of TM_7-PVA_{14}, while the control group received 0.5 ml PBS. The PCA titres were determined on days 30, 37 and 44.

Fig 6. SUPPRESSION OF ESTABLISHED ANTI-TM RESPONSE

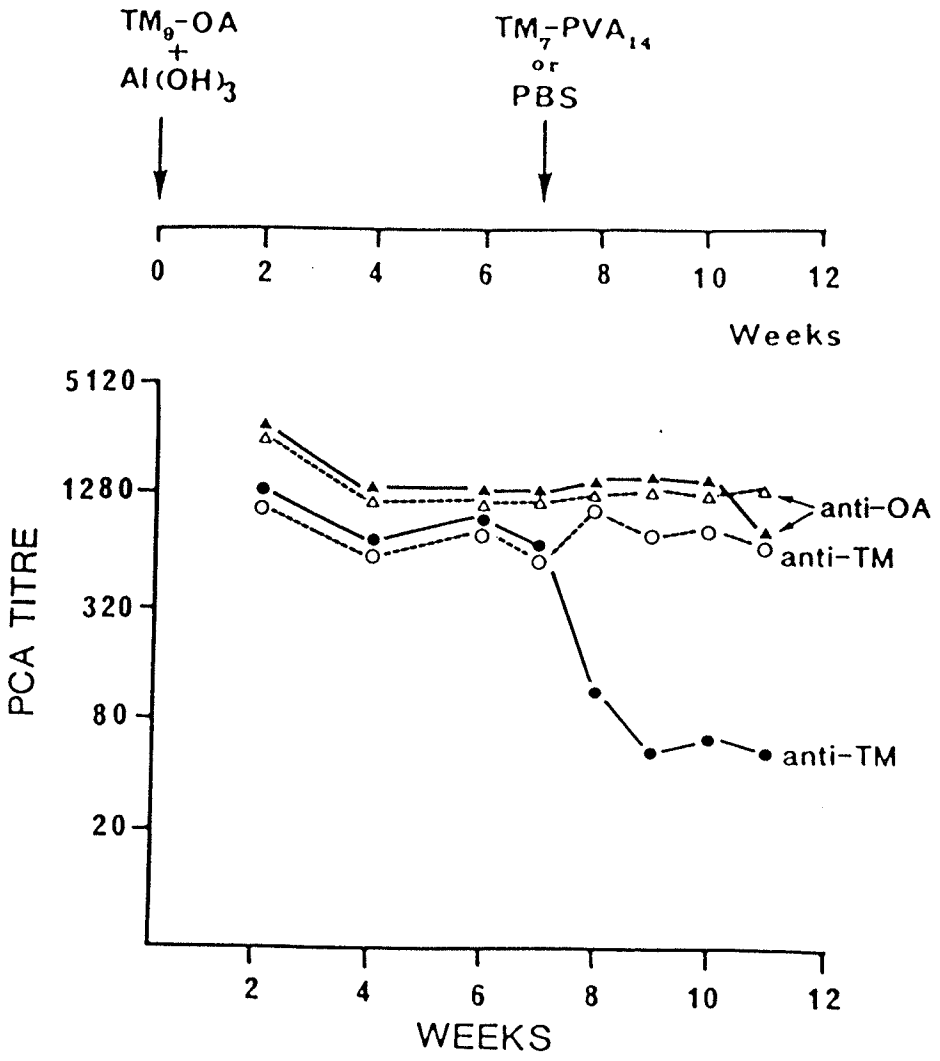


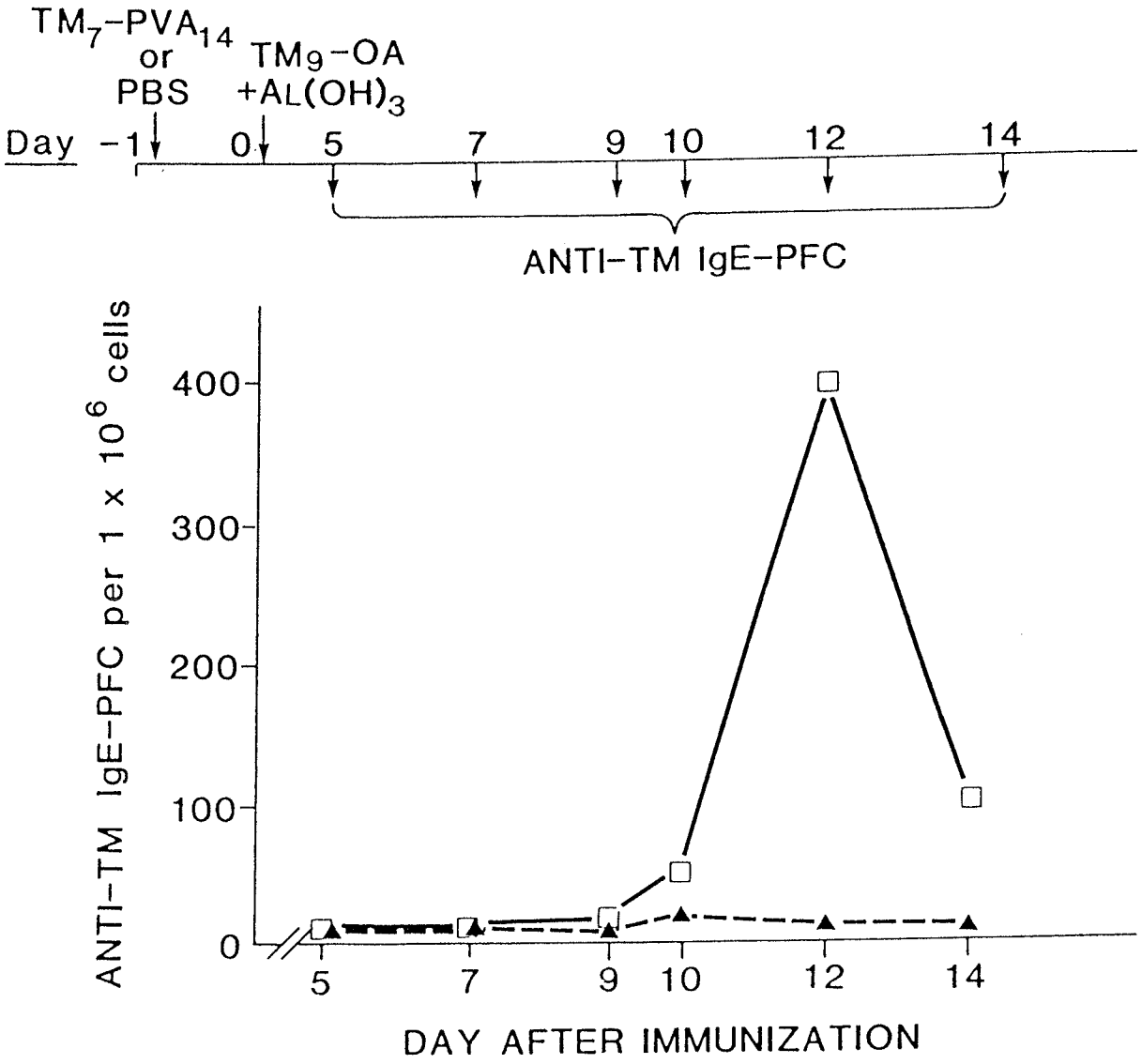
Fig 6. Suppression by TM_7-PVA_{14} of the anti-TM IgE response induced by one sensitizing dose of TM_9-OA. Seven weeks after sensitization the test group received an i.p. injection of 1 mg TM_7-PVA_{14}, and the control group received only PBS. The PCA titres of anti-OA are represented for the test group by (▲ —— ▲) and for the control group by (Δ ---- Δ); PCA titres of anti-TM for the test group (● —— ●) and the control group (○ ------○).

prior to immunization with a sensitizing dose of TM_9-OA. The anti-TM IgE-PFCs were measured afterwards. From the results shown in Fig 7, it may be seen that mice in the control group which did not receive treatment of TM_7-PVA_{14} exhibited a maximum of the anti-TM IgE PFCs around day 12. However, the treatment with TM_7-PVA_{14} prior to immunization almost totally abrogated the anti-TM IgE-PFCs.

5. Comparison of the suppressive effect between TM_7-PVA_{14} and TM-proteins

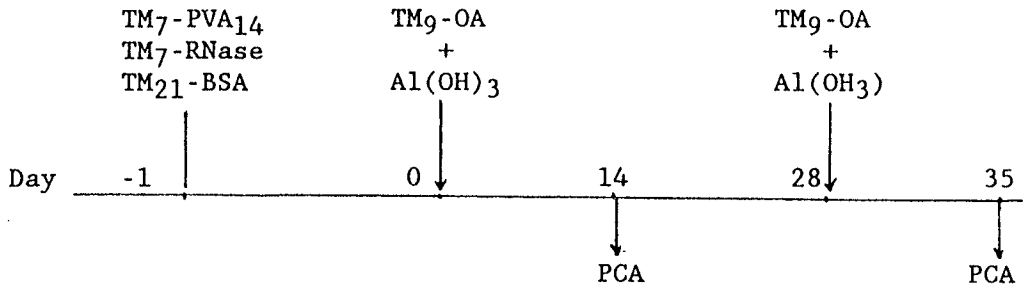
It was reported by several authors (51) that hapten-protein conjugates may also exert a suppressive effect on anti-hapten antibody responses. To compare the suppressive effect of TM-PVA with that of TM-proteins, TM_{21}-BSA and TM_7-RNase were prepared. The TM_7-RNase conjugate had a comparable hapten density and molecular weight to that of TM_7-PVA_{14}. All these conjugates were injected at a dose of 1 mg one day prior to immunization with a sensitizing dose of TM_9-OA, and the mice received a second sensitizing dose of TM_9-OA on day 28. As illustrated in Table 8, TM-BSA and TM_7-RNase could inhibit anti-TM primary IgE response to a certain extent. However, as compared to the suppressive effect of TM_7-PVA_{14}, the suppression by TM-BSA or TM_7-RNase was much weaker, and it almost disappeared after secondary immunization.

Fig. 7 THE SUPPRESSION OF ANTI-TM IgE-PFC



Suppression of anti-TM IgE-PFC. One day before the sensitization with TM_9-OA, the test group (▲----▲) received 1 mg of TM_7-PVA_{14} i.p. and the control group (□——□) PBS.

Table 8. Suppressive effect of TM_7-PVA_{14} and TM-Protein conjugates



	anti-TM PCA titres	
Treatment	Day 14	Day 35
TM_7-PVA_{14}	80	320
TM_7-RNase	710	10,280
TM_{21}-BSA	640	10,280
PBS	2,560	10,280

On day -1 different groups of BDF1 mice received i.p. 1 mg of each the listed conjugates, and on days 0 and 28, all the mice received a sensitizing dose of TM_9-OA. On days 14 and 35 the mice were bled for determination of anti-TM PCA titres.

6. The optimal time interval for induction of tolerance between administration of tolerogen and immunogen

To determine the optimal time interval to induce tolerance, different groups of mice received 1 mg TM_7-PVA_{14} or PBS i.p., and 1, 2, 4, 6 and 8 weeks later they were immunized with a sensitizing dose of TM_9-OA; their PCA titres were measured 14 days after the sensitizing injection. As illustrated in Table 9, that as long as the interval between the treatment and immunization was shorter than 4 weeks, the tolerogen-treated animals still showed more than a 4 fold reduction of anti-TM IgE antibodies as compared to the control group which had received PBS, with highest degree of downregulation being manifested within the first 2 weeks.

7. Prevention of systemic anaphylaxis in sensitized mice by administration of TM_7-PVA_{14}

Although the above findings demonstrated that an established anti-TM response could be profoundly suppressed by different TM-PVA conjugates, there was no evidence that a relationship existed between the rapid decrease in IgE serum titres following administration of these conjugates on the one hand, and the state of tissue sensitivity of the animals on the other. Hence, the experiment illustrated in Fig 8 was designed to investigate the effect of treatment of sensitized mice with tolerogenic conjugates on their systemic sensitivity. For

Table 9. Determination of the optimal interval between injections of tolerogen and immunization

		PCA titres				
		Interval between injections of the tolerogen and immunization				
Treatment		1 w	2 w	4 w	6w	8w
TM_7-PVA_{14}	anti-TM	40	80	220	710	810
	anti-OA	1,410	1,410	2,560	1,010	N.D.
PBS	anti-TM	1,410	1,070	1,410	1,380	1,280
	anti-OA	2,560	1,620	3,020	1,410	N.D.

BDF1 mice were treated with 1 mg of TM_7-PVA_{14}. After 1, 2, 4, 6, and 8 weeks, they were immunized with a sensitizing dose of TM_9-OA and their anti-TM and anti-OA IgE antibody response were determined by PCA 14 days after immunization.

this experiment, the mice were immunized on day 0 with a sensitizing dose of TM_3-OA and the test mice were given an i.p. injection of 1 mg of TM_7-PVA_{14} on day 28; the control group of mice received PBS instead of TM_7-PVA_{14}. On day 30 all the mice received a second sensitizing dose of TM_3-OA in the presence of 8×10^9 Bp organisms; the inclusion of these organisms was necessary to render this strain of mice susceptible to systemic anaphylaxis on further challenge with a polyvalent antigen (115). As is evident from the results given in Fig 8, one i.v. injection of OA, one week after the second sensitizing dose of TM_3-OA, into animals which had received either PBS or TM_7-PVA_{14} resulted in the death of all the animals within 60 minutes after challenge. Similarly, the control group of animals which had not been protected by the administration of TM_7-PVA_{14} died within 90 minutes after challenge with TM_{21}-BSA. In contrast, none of the animals which had received TM_7-PVA_{14} showed any untoward effect of the challenge with TM_{21}-BSA. Hence, it may be concluded that administration of the suppressogenic TM_7-PVA_{14} conjugate resulted not only in the rapid disappearance of circulating anti-TM IgE antibodies, but also in the systemic desensitization of the animals so treated.

Fig.8 PROTECTION OF SENSITIZED MICE FROM SYSTEMIC ANAPHYLAXIS

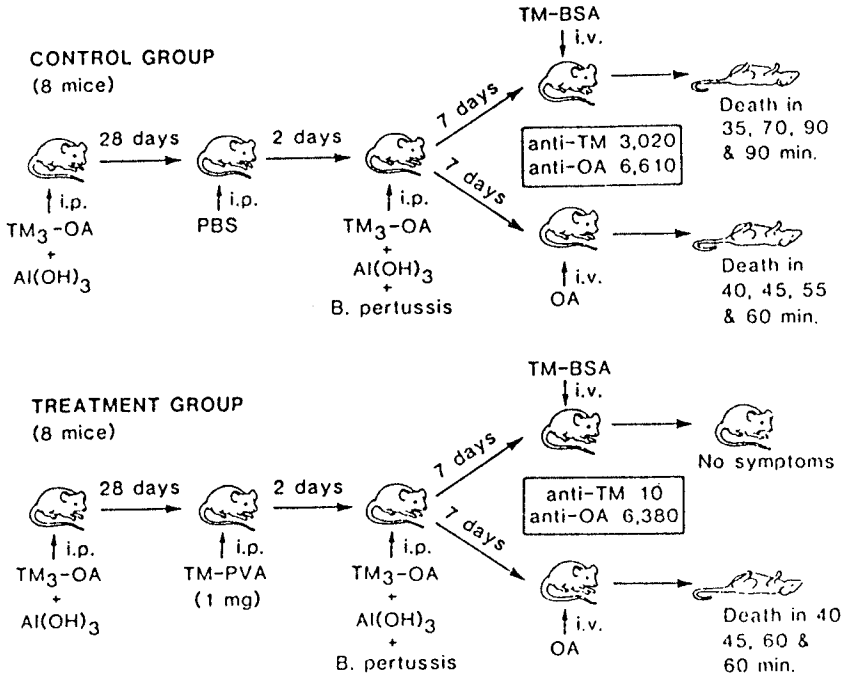


Fig 8. Prevention of TM-specific systemic anaphylaxis by prior treatment with TM_7-PVA_{14}. All the BDF1 mice were immunized on day 0 with a sensitizing dose of TM_9-OA and the test mice were given an i.p. injection of 1 mg TM_7-PVA_{14} on day 28; the control group of mice received PBS instead of TM_7-PVA_{14}. On day 30 all the mice received a second sensitizing dose of TM_3-OA in the presence of Bp. On day 37 the mice were challenged with either 1 mg OA or TM_{21}-BSA.

8. The capacity of TM-PVA and TM-NH-PVA to elicit PCA responses and to induce anaphylactic shock

Since the tolerogenic conjugates consist of TM haptenic groups coupled onto the PVA backbone, it may be envisaged that they can crosslink the IgE molecules fixed on the Fcε receptors on the mast cell membrane, thus causing histamine release.

To test the capacity of different conjugates to elicit PCA responses, volumes of 0.05 ml of serial dilutions of a strong anti-TM serum of known PCA titre were injected on the dorsal skin of hooded rats. After 24 hours, 1.0 ml solutions containing 1 mg of different conjugates and 0.5% Evans' blue were injected i.v. and the PCA titres were determined. As shown in Table 10, the conjugates of lower epitope density had only a weak PCA eliciting capacity, whereas TM_7-PVA_{14} elicited a PCA titre of 230. Thus, as would be expected, it may be concluded that PVA conjugates possessing a higher epitope density, though having a stronger tolerogenicity, had a higher capacity to trigger a local cutaneous anaphylaxis in skin sites sensitized with anti-TM IgE antibodies.

Since it was shown in the above experiment (Fig 8) that injection of TM_7-PVA_{14} before the secondary immunization of TM_3-OA plus Bp could prevent the treated mice from dying of anphylactic shock, the ability of TM-PVA conjugates themselves in inducing anaphylactic shock was tested with a similar protocol. For this purpose, mice were immunized with a sensitizing dose of TM_9-OA in $Al(OH)_3$. After 62 days, mice were bled for PCA determinations and given an injection of B_p 1 x 10^{10}. One

Table 10. The capacity of different preparations of TM-PVA and TM-NH-PVA conjugates to elicit PCA

Challenging conjugates	anti-TM-PCA titre
$TM_{0.5}$-NH-PVA_3	10
$TM_{0.9}$-NH-PVA_3	20
$TM_{1.7}$-NH-PVA_{14}	50
$TM_{1.4}$-PVA_3	100
TM_7-PVA_{14}	230
TM_{15}-BSA	2,820

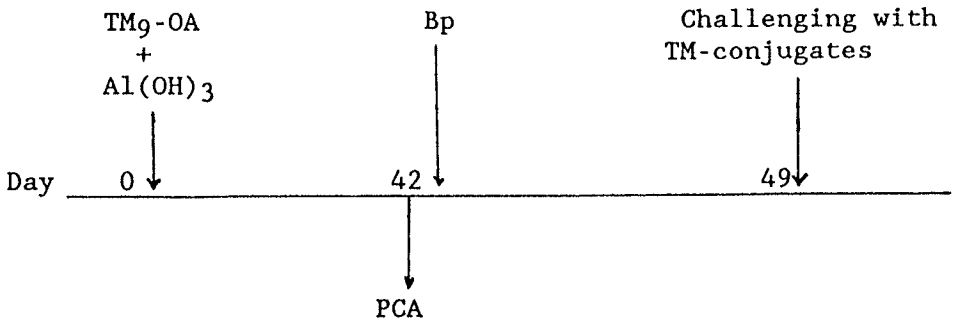
Random bred hooded rats were sensitized i.d. with a mouse anti-TM reaginic serum and received i.v. 24 hours later 1 ml volumes containing 1 mg of the compounds shown, together with 0.5% Evans' blue dye in PBS.

week later, different groups of mice were challenged with TM_{21}-BSA, TM_7-PVA_{14}, $TM_{1.4}$-PVA_3, $TM_{0.5}$-NH-PVA_3, and $TM_{1.7}$-NH-PVA_{14}. As shown in Table 11 , mice challenged with conjugates having low epitope density showed no overt symptoms and none of them died of anaphylactic shock. However, all the mice challenged with TM_7-PVA_{14} showed severe dyspnoea similar to that of mice challenged with TM_{21}-BSA. Although only one mouse in the group challenged with TM_7-PVA_{14} died of anaphylactic shock, as compared to three out of four in the group challenged with TM_{21}-BSA, this difference is not significant ($P=0.2$).

9. <u>The suppression of anti-TM IgE response induced by aerosol inhalation</u>

As mentioned at the beginning of this section, TMA is a chemical which induces readily respiratory symptoms when inhaled. Hence, a mouse model was established, in which the mice were injected i.p. with 1 mg of $Al(OH)_3$, and were exposed to an aerosol of 1% TM_9-OA in a sealed plastics box connected to a nebulizer. When mice were exposed to the aerosol , 1 % TM_9-OA was placed in the nebulizer and the hospital compressed air was introduced into the plastic box (size: 24 x 18 x 16 $inches^3$, with a fan inside to circulate the air to make aerosol spread homogeneously) via a pressure gauge and a nebulizer. The pressure used for aerosolization was 10 psi. From the data in Table 12, it can be seen that under the above condition, mice needed to be exposed to 1% TM_9-OA on day 0 and day 28 and for 30 minutes each

Table 11. The capacity of TM-PVA and TM-NH-PVA to induce anaphylactic shock



compounds used for challenge	symptoms
TM_{21}-BSA	very, sick, severe dyspnoea, 3 died, one survived
TM_7-PVA_{14}	very sick, severe dyspnoea, one died, 3 survived
$TM_{1.4}$-PVA_3	no symptoms, none died
$TM_{0.9}$-NH-PVA_{14}	no symptoms, none died
$TM_{0.5}$-NH-PVA_3	no symptoms, none died

Groups of 4 BDF1 mice each were immunized with a sensitizing dose of TM_9-OA and on day 42 they were bled and injected with 1×10^{10} *Bordetella pertussis* (Bp). On day 49 these mice were challenged i.v. with 1 mg of the conjugates listed above. The PCA titre of the serum collected on day 42 was 640.

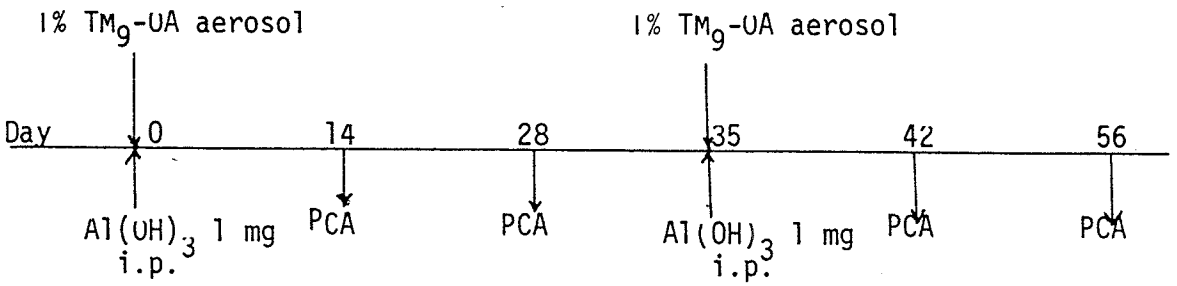
time to reach a reasonable secondary anti-TM IgE response. In the following experiment, the BDF1 mice were exposed to 1% TM_9-OA for 30 minutes on days 0 and 28. The treatment group was injected on day 27 with 1 mg TM_7-PVA_{14} , while the control group was injected PBS. From the results in Table 13, it was evident that BDF1 mice treated with TM_7-PVA_{14} 1 mg i.p. prior to the secondary immunization developed no detectable anti-TM IgE response, while the control group still developed some anti-TM IgE response. Thus, it was concluded that TM-PVA conjugates could suppress anti-TM IgE responses which were elicited by aerosol inhalation.

10. The suppression of secondary anti-TM IgE response in MAXX rats

The tolerance-inducing ability of $TM_{0.9}$-NH-PVA_3 was also tested in rats to serve two purposes: if they may represent a good model for investigations of bronchial asthma and to establish whether or not TM-PVA treatment is effective in another species of animals.

In the preliminary experiment, MAXX rats were found to be good IgE responders to TM-OA (data not shown). In the following experiments, 12 MAXX rats were divided into 4 groups of 3 rats each. On day 0, Groups I and II received TM_9-OA 100 μg in the presence of $1x10^{10}$ Bp into footpads, whereas Groups III and IV were immunized in footpads with 100 μg of TM_6-OA plus $1x10^{10}$ Bp. All the rats were bled on day 10, day 25 and day 35. On day 35, Groups I and III were injected with $TM_{0.9}$-NH-PVA_3 2 mg i.p., whereas Groups II and IV were treated only

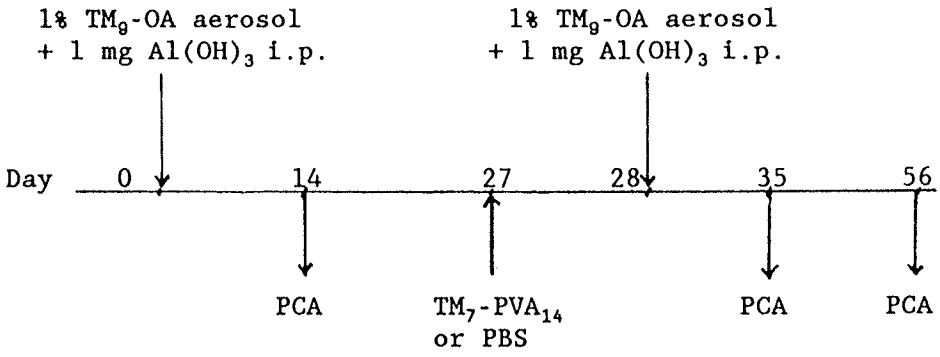
Table 12. Induction of murine IgE response by aerosol inhalation



Time of aerosoliation	PCA									
	Day 14		Day 28		Day 35		Day 42		Day 56	
	anti-TM	anti-OA	anti-TM	anti-OA	anti-TM	anti-OA	anti-TM	anti-OA	anti-TM	anti-OA
5 min	< 2	< 2	< 2	N.D.	< 2	1,820	< 2	N.D.	< 2	N.D.
10 min	< 2	< 2	< 2	N.D.	32	1,660	< 2	N.D.	< 2	N.D.
30 min	< 2	128	< 2	N.D.	64	710	80	N.D.	80	N. D.

BDF1 mice were injected i.p. with 1 mg $Al(OH)_3$ on day 0 and 28, and on the same days they were exposed to 1% TM_9-OA aerosol for 5, 10 or 30 minutes. The mice were bled on days 14, 28, 35, 42 and 56 for PCA determination.

Table 13. The suppression of anti-TM IgE response induced by aerosol inhalation



Treatment	PCA					
	Day 14		Day 35		Day 56	
	anti-TM	anti-OA	anti-TM	anti-OA	anti-TM	anti-OA
TM_7-PVA_{14}	<2	20	<2	1,280	<2	710
PBS	<2	40	64	1,320	32	640

On days 0 and 28 BDF1 mice were i.p. injected with 1 mg of $Al(OH)_3$ and inhaled 1% TM_9-OA for 30 minutes. On day 27, the test group was injected with 1 mg of TM_7-PVA_{14}, while the control group was injected PBS. On days 14, 35 and 56, the mice were bled for IgE determination.

Table 14 The suppression of secondary anti-TM IgE response in MAXX rats

	1st Response						2nd Response					
	Day 15		Day 25		Day 35		Day 38		Day 40		Day 42	
antigen	anti-TM	anti-OA	anti-TM	anti-OA	anti-TM	anti-OA	anti-TM	anti-OA	anti-TM	anti-OA	anti-TM	anti-OA
TM_6-OA	20	< 10	< 10	< 10	< 10	< 10	20	20	50	250	< 10	30
Test group (I)	50	10	20	20	20	40	20	100	40	380	< 10	100
	210	50	70	150	< 10	150	110	360	40	420	20	260
Control group (II)	220	30	60	20	70	80	90	260	140	360	70	100
	60	20	60	20	70	< 10	320	100	140	430	70	100
	20	10	20	< 10	< 10	< 10	60	30	130	240	30	20
TM_6-OA	230	150	< 10	70	20	60	20	230	50	330	< 10	70
Test group (III)	20	10	10	10	10	20	20	90	40	360	< 10	70
	130	120	20	90	20	110	40	450	30	420	< 10	250
Control group (IV)	60	10	40	< 10	30	< 10	150	110	330	230	30	30
	20	10	30	< 10	40	< 10	150	100	330	230	30	30
	20	10	70	20	40	< 10	280	230	290	420	30	70

MAXX rats were immunized by injection of 100 ug of TM_6-OA or TM_9-OA plus 1×10^{10} BP into footpads. On day 35, the test groups were injected i.p. with 2 mg of $TM_{0.9}$-NH-PVA_3 whereas the control groups received only PBS. On day 35, all the rats were boosted i.m. with 100 ug of homologous antigen without adjuvant. On days 15, 25, 35, 38, 40, and 42, the rats were bled for PCA determinations.

with PBS. On the same day all the rats were reimmunized i.m. with the 100 μg homologus antigen, and bled on days 38,40 and 42 for determination of the secondary IgE responses. From the results in Table 14, it may be seen that although there were some variations of antibody titres within the same group of rats, the antibody titres of the secondary responses in the same group were much more consistent, i.e., Groups I and III had much lower secondary anti-TM IgE antibody titre than the corresponding control groups. However, the anti-OA IgE response was comparable in both the test and control groups.

Discussion

As stated earlier, inhalation of TMA was shown to be implicated in 4 different pulmonary syndromes, of which 3 (rhinitis and asthma, LRSS, and pulmonary edema and hemorrhage with anemia) may be caused by antibodies against conjugates of TMA with the patients' proteins or erythrocytes (95-100). In the experiments described above, it was demonstrated that PVA conjugates with TMA suppressed not only the induction of the primary anti-hapten humoral response in BDF1 mice manifested by antibodies of the IgE and other classes but, more importantly also the ongoing anti-TM response in presensitized mice and MAXX rats. Furthermore, in agreement with the results of the earlier studies of the effects of BPO- and DNP-PVA , TM-PVA conjugates suppressed exclusively the anti-hapten responses without affecting the anti-carrier responses. The effectiveness of TM-PVA conjugates was

dose and epitope density dependent as demonstrated by the fact that conjugate at low dose or low epitope density exerted a lower inhibitory effect. With regard to conformational aspects, it is interesting to note that spacer, such as hexanediamine did not affect the tolerogenicity of the conjugates. In fact, TM_7-PVA_{14} conjugates, prepared by coupling TMA directly onto PVA, seemed to have a stronger tolerogenic activity. This is probably due to the higher epitope density of this conjugate. Moreover, TM_7-PVA_{14} was not only effective in suppressing the humoral anti-TM immune responses induced by i.p. injection of antigens, but also in preventing the anti-TM IgE induced by aerosol inhalation .

Compared with DNP-PVA conjugates of the same epitope density, the TM-NH-PVA or TM-PVA conjugates seemed to be less effective in inducing specific tolerance. Patterson *et al* (101,102) demonstrated that the "anti-TM" human antibodies were actually directed against new antigenic determinants on patients' modified proteins which encompassed also the TM group . Hence, it may be visualized that tolerogen may bind with only low affinity to the antigen binding receptors on the target cells, either B or T cell. Only at higher epitope density could the conjugates have enough avidity to interact with the target cells to induce effective tolerance.

Because the Fc receptors of mast cells and basophils have a high affinity for IgE antibodies, the IgE antibodies produced may first saturate the receptors on the mast cells and basophils before appearing in the serum. In support of this concept, one may quote the results of Vaz *et al* (6) who had shown that in some mice which did not

have demonstrable serum reagin , substantial mast cell sensitization had occured. Therefore, the IgE titre in the serum probably may not necessarily reflect the real tissue sensitivity. In accordance with earlier results in this Laboratory that BPO-PVA conjugates could desensitize the presensitized animals (92), TM_7-PVA_{14} conjugate were also shown to protect the animals against systemic anaphylaxis. Several possibilities might explain the apparent desensitization mechanism within this 9 day interval between second sensitizing injection and challenge of the mice with polyvalent hapten conjugates: (1) Assuming that anti-TM IgE is a large fraction of the total IgE, the re-equilibrium between the membrane and liquid phase IgE after tolerogen injection might lead to the dissociation of specific IgE from the cell surface. Because of the short half-life period of circulatory IgE and its possible neutralization by polyvalent tolerogen, the animals would be no longer susceptible to systemic anaphylaxis. (2) Being a polyvalent conjugate itself, TM_7-PVA_{14} could desensitize the mast cells by slowly releasing the mediators, thus preventing the mice from extreme anaphylactic shock even after receiving Bp for rendering them highly sensitive to histamine .

As had been documented by Urban *et al* (17) when IgE is expressed on the B cell surface, these cells are committed for IgE production, which is a T-independent process. However, the further differentiation of IgE bearing cells to IgE-forming cells is a T-dependent process. Approaches which have been used to suppress specific IgE responses involved either the inactivation of the immature B cells or the

expansion of Ts cells responsible for the downregulation of B cell differentiation. Thus as demonstrated (*24*), the effect of DNP-DGL conjugates is to inactivate hapten specific B cells without the generation of detectable hapten specific Ts cells . Klinmann *et al* (116) have shown that whereas DNP-DGL could dramatically abrogate the ongoing anti-DNP response in mice, it would only eliminate immature B cells both *in vivo* and *in vitro* at a specific stage in clonal maturation, i.e., when these cells were first expressing antigen-binding receptors; and DNP-DGL had no efect on mature resident B cells. As most B cells appear to be short-lived and have a half-life from 4 days to 2 weeks, no new hapten-specific B cells would be generated in the presence of this tolerogen.

It was shown by Okudaira (10) and in this Laboraotory (8,9) that long-lived IgE forming cells were responsible at least in part for the persistent IgE response in mice. However, as demonstrated earlier (91), DNP-PVA conjugates not only inactivated hapten-specific B cells, but also induced hapten-specific Ts. On the other hand, in this study, the author was not able to demonstrate the induction of Ts cells by TM-PVA conjugates. Therefore, some PVA conjugates may differ in their effects on the different subpopulation of the immune system. In this connection, it is interesting to note that although 7 weeks after a single injection of TM_9-OA, TM_7-PVA_{14} significantly reduced the specific IgE response, it did not obliterate completely the anti-TM IgE response. Several theoretical possibilities may be offered to explain the persistence of the IgE response: 1) The hapten-PVA conjugate may be able to block primarily, if not

exclusively, the long-lived IgE forming cells; 2) TM_7-PVA_{14} may inactivate only the B cells with high affinity receptors, the cells with low affinity escaping inactivation; 3) The long-lived IgE forming cells may contribute to the IgE production only to a minor extent. Taking an overview of these possibilities, it may be hypothesized that TM_7-PVA_{14} conjugates had no effect on long-lived antibody-forming cells either because of their developmental stage or because of the low affinity of their receptors. However, as new recruitment of B cells appears to be prevented in the presence of this tolerogen, the net effect of administration of these conjugates is a marked decrease of specific IgE level. The experiments described in the next chapter were designed with a view to test the effect of TM_7-PVA_{14} conjugates on the persistent IgE responses.

II. The suppression of the radioresistant IgE response

In allergic individuals, as well as in certain strains of mice immunized with low doses of antigen in $Al(OH)_3$, production of IgE antibody continues over protracted periods of years or months in the absence of an overt exposure to the allergen. It has been demonstrated that in BDF1 mice the anti-OA IgE response induced by minute quantities of OA in $Al(OH)_3$ could persist for over one year (8,9). Since the half-life of mouse IgE in circulation is only 10.5 to 12.5 hours (9,118), the maintenance of a steady state of IgE antibody must involve a continued IgE production. The basis for this long-term IgE production is of considerable interest and of particular importance in developing therapeutic strategies for shutting off its production. Several mechanisms have been proposed: 1) The antigen in $Al(OH)_3$ forms a depot in the peritoneal cavity of the mouse and is slowly released to stimulate the hosts' immune system (119). 2) As proposed by Tew *et al* (117), lymphocyte follicles may store antigen in an immunogenic form, i.e., in the form of immune complexes bound to lymphoid cells via Fc receptors; accordingly, when concentration of free antigen drops in circulation, the complexes dissociate and the antigen is released into the circulation, which continues to stimulate the immune system. 3) In the mouse, IgE was produced by long-lived, radioresistant IgE-forming cells (8,9,10); and 4) B cells may be stimulated by anti-id antibodies and/or id-specific Th cells (41).

Results

1. Persistent IgE response is radioresistant

In an exploratory experiment, it was established that about one month after the primary immunization with a sensitizing dose of TM_9-OA, the IgE antibody level declined to a plateau and was essentially not affected by a dose of 1,000 R of whole body irradiation. However, irradiation resulted in the suppression of the host's ability to mount a secondary anti-TM and anti-OA IgE antibody response to the homologous antigen, which was interpreted to indicate that the memory B cells were either deleted or prevented from further development (data not shown).

2. Persistent IgE response was not produced by substituted bone marrow cells

To establish if the prolonged radioresistant IgE response might be also due to the continuing stimulation of the immune system by the antigen, the following experiment was designed. The BDF1 mice were immunized i.p. with 1 μg or 10 μg TM_9-OA in the presence of 1 mg $Al(OH)_3$. One day later, they received whole body irradiation (1,000 rads) and were subsequently infused with 2×10^7 bone marrow cells from normal syngeneic animals. Beginning two weeks after the immunization, the mice were bled weekly for 8 weeks. No PCA titres of either anti-TM or anti-OA were detected in these animals. Hence, these

results support the view that the radioresistant persistent IgE response was not likely due to the continued stimulation of newly differentiated bone marrow cells by residual antigen.

3. Radioresistant persistent IgE response is sensitive to the treatment with TM_7-PVA_{14}

As state earlier, the prolonged IgE response was shown to be relatively refractory to the effect of Ts cells (12,14). However, Okudaira *et al* (10) reported that the persistent anti-DNP IgE response, induced by DNP-OA, could be inhibited by DNP_7-MGG both *in vivo* and *in vitro*. Therefore, the effect of tolerogenic conjugates of TM-PVA on the radioresisant component of the IgE response was tested by the protocol illustrated in Fig 9.

The BDF1 mice in the test and control groups were immunized on day 0 with TM_9-OA in $Al(OH)_3$ and the level of circulating IgE was determined in all mice 18 weeks thereafter. One day later, Groups I and II received whole body irradiation (1,000 Rads), while Groups III and IV received no irradiation. On the subsequent day Groups II and IV received 1 mg of TM_7-PVA_{14}, whereas Groups I and III only PBS. At a further interval of one day, $1x10^7$ bone marrow cells were transferred from normal syngeneic animals into each of the mice in the four groups; this treatment was essential to minimize mortality among the irradiated mice.

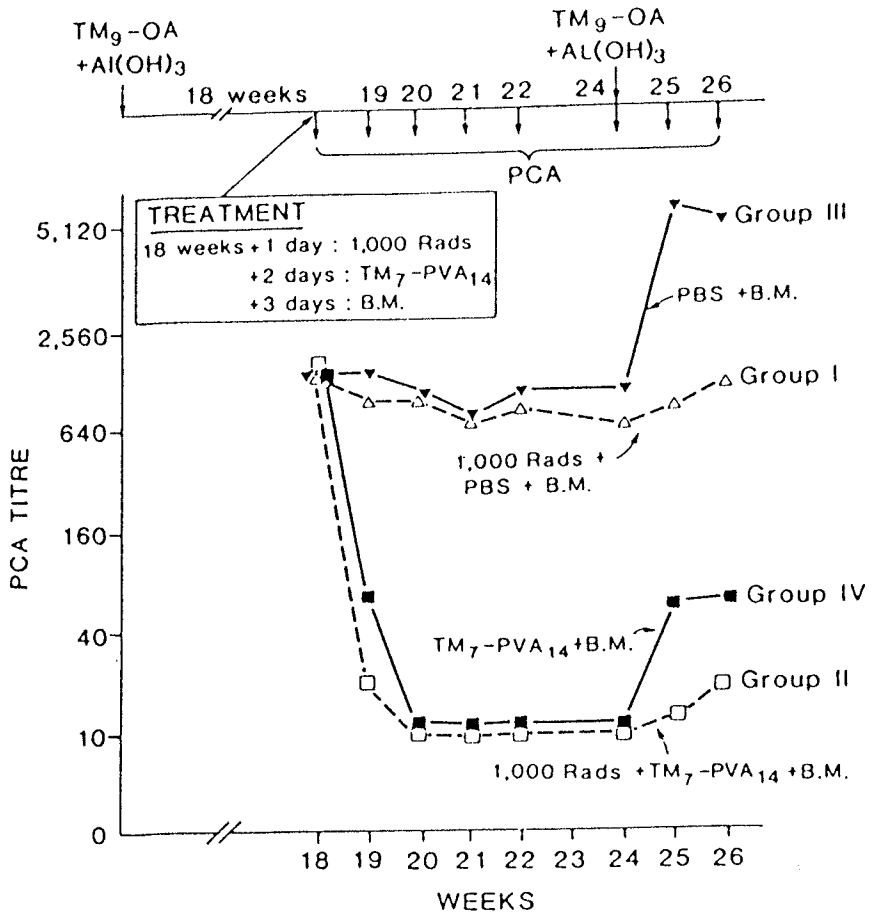


Fig 9. The effect of TM_7-PVA_{14} on the radioresistant IgE response. The treatment of the mice was begun 18 weeks after sensitization as shown in the inset. Group I (△----△) and Group II (□----□) received whole body irradiation (1,000 Rads), while Group III (▼——▼) and Group IV (■——■) received no irradiation. One day later Groups II and IV received 1 mg of TM_7-PVA_{14} and Groups I and III only PBS. After a further interval of one day, all the mice were infused with 1×10^7 bone marrow cells from normal syngeneic animals. Six weeks later all the mice were immunized with a second dose of TM_9-OA. PCA titers were determined at weekly intervals.

As is evident from the results plotted in Figure 9, bone marrow cells did not affect the protracted IgE level in the control Group III. From a comparison of PCA titres of Groups I and III, it is also evident that the high dose of irradiation had almost no effect on the established IgE response. However, this radioresistant phase of the anti-TM IgE response was nearly completely abrogated by the administration of TM_7-PVA_{14} for at least 6 weeks, at which time all mice received a booster injection of the sensitizing dose of TM_9-OA. As suggested by the data shown in Figure 9 , this second injection of TM_9-OA (6 weeks after irradiation) did not elicit a sudden rise of PCA titres in the irradiated Groups I and II, which may be interpreted as indicating that irradiation had prevented the recruitment of B memory cells. Since Th cells are radioresistant (24,41), lack of a secondary IgE response in the irradiated mice could be interpreted as inactivation or deletion of B memory cells. As in the previous experiments, it ought to be stressed that the anti-OA IgE response of none of the four groups of mice was affected by the treatment of TM_7-PVA_{14} (data not shown).

4. Confirmation of deletion of B memory cells by irradiation

To further confirm that whole body irradiation of such a high dose indeed prevented the function of memory B cells, the following experiment was designed. In this experiment, the BDF1 mice were immunized with a sensitizing dose of TM_9-OA. After 40 days, the mice

were irradiated with a dose of 1,000 R and then infused with bone marrow cells from normal syngeneic animals. Two weeks after irradiation, the spleen cells were collected from these mice and transferred into groups of syngeneic mice which had been sublethally irradiated with 650 R. The recipient mice were then challenged with a sensitizing dose of TM_9-OA. From the results shown in Table 15, it is evident that the cells of mice of the first control group, which had not been irradiated but had been immunized, gave rise to an extremely high secondary IgE response in the recipients to both TM and OA 7 and 14 days after the booster injection of the sensitizing dose of TM_9-OA. By contrast, cells of the irradiated donors mounted only a relatively weak IgE response in response to the booster immunization. However, cells from normal nonirradiated donors were not stimulated to mount an IgE response to either TM or OA in the sublethally irradiated recipients.

5. The *in vitro* effector-blockade by TM_7-PVA_{14} of antibody forming cells

The effect of TM_7-PVA_{14} conjugates on antibody-forming cells was investigated using the experiment described below. Groups of four mice each were immunized with a sensitizing dose of TM_9-OA and sacrificed 12 days later. Their spleens were pooled for the preparation of a single cell suspension. Aliquots of this suspension were incubated with TM_7-PVA_{14} at different concentrations at 37°C for 60 minutes and then washed three times with cold MEM medium prior to testing for IgE-PFC. It is evident from the results listed in Table 16 that the incubation

Table 15. Deletion of B memory cells by lethal irradiation

Groups	Spleen cells of donors	Irradiation of donors	PCA			
			Day 7		Day 14	
			anti-TM	anti-OA	anti-TM	anti-OA
test group	TM_9-OA primed	1,000R	160	320	160	640
controlI	TM_9-OA primed	none	> 12,800	>12,800	>12,800	>12,800
Control II	"naive"	none	<10	<10	<10	<10

Donor mice were immunized with a sensitizing dose of TM_9-OA and after 40 days some of them were irradiated with 1,000 R. Two weeks later they were sacrificed and their spleen cells were passively transferred (4×10^{10}/mouse) into 650 R sublethally irradiated syngeneic BDF1 mice. The cells of the second control were provided by donors which had not been immunized, referred to as "naive". The recipients were immediately immunized with a sensitizing dose of TM_9-OA.

Table 16. Effector blockade by TM_7PVA_{14} of plaque formation

TM_7-PVA_{14} (μg/ml)	PFC 5 x 10^5 cells
0	71
1	26
4	14
16	6
63	2
250	2

Mice were immunized with a sensitizing dose of TM_9-OA; 12 days later their spleen cells were incubated with TM_7-PVA_{14} at different concentration at 37°C for 1 hour. The cells were washed 3 x and tested for PFC.

with TM_7-PVA_{14} resulted in significant reduction, in a dose dependent manner, of the ability of these cells to secrete antibody. In a separate experiment illustrated in Table 17, it was demonstrated that TM_7-PVA_{14} suppressed the anti-TM IgE PFC, but had no effect upon anti-DNP IgE PFC or anti-SRBC IgM PFC even at a concentration of 250 μg/ml. Hence, it was concluded that TM_7-PVA_{14} even at this relatively high dose had no toxic effect upon antibody forming cells and that the suppressive effect was hapten-specific.

6. The suppression of *in vivo* and *in vitro* antibody secretion by anti-NP IgE producing hybridoma cell line #1588 by NP-PVA

In order to determine the suppressive effect of hapten-PVA conjugates on the secretion of antibodies by antibody forming cells, a hybridoma cell line #1588 producing anti-NP IgE antibodies was used in conjunction with NP_4-PVA_{14}. This cell line was provided by Ms J Fisher in the Hybridoma Unit of the MRC Group for Allergy Research of this Department. A dose of $1x10^6$ NP-specific IgE producing hybridoma cells (CB_6F1 x BALB/c) was injected i.p. into CB6F1 male mice , which had been pretreated with an injection of 0.5 ml of pristan i.p. 1 week previously. Ten days after inoculation of the hybridoma cells, the test group received i.p. 1 mg of NP_4-PVA_{14} (synthesized by Mr. Stephan Krueger of this Department), while the control group was injected with PBS only. From the results listed in Table 18, it is evident that the test group consistently had lower circulatory anti-NP

Table 17. Specificity of inhibition of anti-TM PFC by TM_7-PVA_{14}

	PFC per 1 x 10^6 spleen cells*		
	TM IgE-PFC	DNP IgE -PFC	SRBC-PFC
cells + TM_7-PVA_{14} (250 μg/ml)	2	193	27
cells + PVA_{14} (250 μg/ml)	114	226	N.D.
cells + MEM	112	263	25

Groups of mice were immunized with a sensitizing dose of TM_9-OA or DNP_3-OA, or sheep red blood cells and sacrificed 12 or days later as shown above. The spleen cells of each of these three groups of mice were incubated, respectively with TM_7-PVA_{14}, PVA_{14}, and MEM at 37°C for 1 hour. The cells were washed 3 x before assay for PFC.

Table 18. Suppression of the secretion of anti-NP IgE producing hybridoma cells *in vivo* by NP_4-PVA_{14}

Groups of mice	PCA titres (anti-NP)			
	Day 14	Day 21	Day28	Day 35
Test group	<40	<40	2,560 (one dead)	5,120 (two dead)
control group	<40	640	20,480 (two dead)	20,480

A dose of 1 x 10^{10} anti-NP IgE producing hybridoma cells were injected i.p. into CB6F1 male mice which had received 0.5 ml pristan one week earlier. Ten days after inoculation of tumor cells, the test group was given 1 mg NP_4-PVA_{14} i.p., whereas the control group was injected with PBS. The mice were bled on different days to test their levels of circulating IgE antibodies by PCA.

IgE antibodies in circulation by comparison to those of the control group. However, since the life span of hybridoma bearing animals was limited, further *in vivo* experiments were abandoned.

The *in vitro* IgE-PFCs formed by the hybridoma cell line #1588 were also inhibited by NP_4-PVA_{14} . When these hybridoma cells (800 cells) were plated in Cunningham chambers with NP-BSA coated SRBC and anti-ϵ developing serum at 37°C for 3 hours, about 50% of the hybridoma cells were detected as NP-specific IgE-PFCs. However, after incubation of the cells with NP_4-PVA_{14} at a final concentration of 10 μg/ml at 37°C for 1 hour, the number of NP-specific IgE PFCs was found to be inhibited by about 80%, i.e., 62± 3 PFCs as compared with 375±32 PFCs. (Prior to plating, the cells were washed 3 times with MEM). After incubation with NP_4-PVA_{14} the hybridoma cells seemed to be in a healthy condition, as determined by the dye-exclusion test.

Discussion

The sustained production of IgE antibody is a feature of immediate type hypersensitivity . The half-life of murine IgE in circulation is only about 10.5 to 13 hours as determined by PCA (9) or by isotope labelling methods (118). However, small doses of antigen in combination with $Al(OH)_3$ can induce persistent IgE responses similar to those described in man. The cellular basis of this persistent IgE antibody response has as yet not been completely elucidated, but experimental evidence suggests that long-lived IgE secreting cells may

be involved. It may be argued that residual antigen or anti-id antibodies may cause the sustained IgE response. However, if residual antigen were indeed responsible for the persisting IgE level, it would be expected that a large dose of antigen would induce a prolonged IgE antibody response, since in such a situation more residual antigen would be retained in the host. As mentioned earlier, to induce a protracted IgE response in appropriate strains of mice, one important condition is to use minute dose of antigen. The finding in the above experiment that the injection of 1 or 10 μg of antigen in the presence of $Al(OH)_3$ before irradiation did not elicit any detectable amount of IgE response in a long period of time also contradicted the above hypothesis.

The putative long lived IgE forming cells are characterized not only by their long life span, and radioresistance, but also by their poor inhibition by Ts cells (11). Hence, the capacity to downregulate these cells has special significance for the development of an effective therapeutic regimen for allergic conditions. In the experiment described above, it was shown that both the protracted anti-hapten and anti-carrier IgE responses were hardly influenced by a dose of 1,000 R of whole body irradiation, which was also shown to result in the impairment of the recruitment of B memory cells (Table 15). Even though the mechanism of the sustained IgE response is still unclear, it should be stressed that the radioresistant anti-TM IgE response was almost totally suppressed for at least 6 weeks by the i.p. adminstration of TM_7-PVA_{14}.

In other experiments focusing on the effects of TM_7-PVA_{14} on

antibody forming cells, it was shown that TM-primed spleen cells lost their capacity to produce PFCs after incubation with TM_7-PVA_{14} (Table 16 & 17). Schrader & Nossal (52,54) reported a few years ago a phenomenon which they termed effector cell blockade and which referred to the specific inhibition of plaque formation by multivalent antigens. Using a hybridoma cell line, they reported that the inhibition was accompanied by aggregation of antigen on the cell surface. Clearance of the antigen from the cell surface correlated with recovery. Watanabe *et al* (120) recently reported that conjugates of hapten with mouse IgG (DNP-MGG), which induced tolerance of normal B cells to DNP (61), also induced inhibiton of DNP-hybridoma PFC *in vivo* when the conjugate was injected during the first two days after *in vivo* transfer; this period coincides with the eclipse phase of hybridoma cell transfer *in vivo*. Administration of tolerogen beyond this period failed to give suppression. Hence, it may be inferred that normal plasma cells at an early stage may possess surface immunoglobulins and that these cells would be subject to antigen modulation. These results agree with the findings of Okudaira *et al* (10) who showed that long lived anti-DNP IgE forming cells were inhibited by multivalent antigen DNP-BGG both *in vivo* and *in vitro*.

In this study, NP_4-PVA_{14} was shown not only to tolerize normal animals, but also to inhibit *in vivo* the secretion of anti-NP specific antibodies from hybridoma cell line even ten days after implantation of the cells (Table 18).

In conclusion, it may be stated that although the mechanism of the persistent IgE production has not yet been fully elucidated, the

experimental data demonstrate that the radio-resistant long-term IgE production could be abrogated in the mouse by hapten-PVA conjugates. One probable mechanism of suppression may involve the blockade of antibody-forming cells.

III. Distribution of hapten-PVA conjugates in the host

On the basis of the results presented so far, the characteristics of the tolerance induced by TM_7-PVA_{14} can be summarized as follows: (1) specific suppression of the humoral anti-TM response could be induced in both naive and sensitized animals, (2) the tolerance was highly hapten-specific, (3) the tolerant state was relatively long-lasting, (4) the persistent, radioresistant IgE response was also sensitive to TM_7-PVA_{14} treatment, (5) TM_7-PVA_{14} could protect TM-sensitized animals from systemic anaphylactic shock.

In view of the potential clinical usefulness of tolerogenic conjugates, such as TM_7-PVA_{14}, it was deemed appropriate to establish its *in vivo* distribution and its clearance from the host; these two aspects of the "bioavailability" of the tolerogen are described below.

1. The clearance of TM_7-PVA_{14} conjugate from circulation

The clearance rate for TM_7-PVA_{14} from circulation was determined from measurement of the levels of TM_7-PVA_{14} in the blood by RAST inhibition (see MATERIALS AND METHODS). For this purpose, 1 mg of TM_7-PVA_{14} was injected i.p. into normal BDF1 mice and at different time intervals, the TM_7-PVA_{14} content of their serum was calculated in terms of its capacity to inhibit the RAST. From data listed in Table 19 , it is obvious that 24 hours after the injection of TM_7-PVA_{14} the concentration of TM_7-PVA_{14} in circulation was below the limit of detection by the inhibition of antibodies in the RAST. For the 5 higher concentrations given in Table 19, a half-life of 1.4 hours was calculated for the elimination of the tolerogen from circulation.

Table 19. Detection of TM_7-PVA_{14} in the circulation by RAST inhibition

Time (hours)	TM_7-PVA_{14} in serum (μg/ml)	$\log_{10}$Con
0.5	47	1.672
2	56	1.763
4	8.8	0.9444
6	2.2	0.3010
8	2	0.3010
12	1.8	0.2553
24	0.35	-0.4559

Since concentrations below 2 μg/ml were beyond the limit of measurements deducible from the "standard" calibration line (see Fig 3), only the first 5 points were used to construct a regression line, in which n=5,
intercept = 1,910
slope = -0.22
$\log_{10} Y = 1.910 - 0.22 X$
r (correlation coefficient) = -0.9492 ($P<0.05$)
if $Y_1 = 50$, $X_1 = 0.96$
if $Y_2 = 100$, $X_2 = 2,32$
Thus $X_{1/2} = 1.4$ hours

2. The *in vivo* distribution of radioactively labelled NIP_4-PVA_{14}

In the above experiment, the distribution of TM_7-PVA_{14} among the organs of the mice was not investigated, since it was not easy to prepare radiolabelled TM-PVA. Hence, ^{125}I-labelled NIP-PVA_{14} was used instead on the assumption that all hapten-PVA conjugates may have similar *in vivo* distribution patterns. The preparation of ^{125}I-labelled NIP-PVA_{14} from NP-PVA_{14} by the chloramine T method is described under MATERIALS AND METHODS

The mice received i.v. 1.75×10^6 cpm of $N^{125}IP_7$-PVA_{14} (estimated to be ca. 2 μg) mixed with 100 μg of cold NP_4-PVA_{14}. Starting one week before the experiment, the mice were given water containing KI (2 g/*l*) throughout the experiment in order to block any possible uptake of radio-iodine by the thyroid. After different time intervals the mice were bled and then sacrificed, and their organs were subjected to radioactive counting. From Table 20, it may be seen that, after injection of the radioactive conjugates, the concentration of the conjugates dropped rapidly in circulation during the first 24 hours, which is in accord with the results described in the preceding section. However, the most significant observation was that the material was retained for extend periods in all organs tested and longest in the kidney and liver. Even after 100 days there was still some detectable amount of $N^{125}IP$-PVA_{14} in the liver.

Table 20. Distribution of $N^{125}IP\text{-}PVA_{14}$ in mice as indicated by C.P.M.

Time after i.v. injection	Blood (0.1ml)	Heart & Lung	Spleen	MLN & Thymus	Kidney	Liver
20 min	2.8×10^4	3.1×10^4	1.5×10^3	1.5×10^3	3.8×10^4	3.9×10^5
24 hr	1.4×10^3	3.5×10^3	2.2×10^3	820	2.2×10^4	4.9×10^5
Day 4	500	2.0×10^3	2.2×10^3	440	1.4×10^4	4.0×10^5
Day 7	200	1.3×10^3	2.6×10^3	410	8.2×10^3	2.5×10^5
Day 12	80	610	1.8×10^3	480	4.8×10^3	8.5×10^4
Day 14	70	600	1.8×10^3	280	4.7×10^3	7.0×10^4
Day 20	70	370	1.4×10^3	290	2.8×10^3	3.9×10^4
Day 28	30	390	1.4×10^3	270	2.8×10^3	3.9×10^4
Day 40	N.D.	250	1.0×10^3	130	1.0×10^3	1.5×10^4
Day 90	10	79	7.5×10^2	150	680	6.6×10^3
Day 100	0	0	85	0	840	2.0×10^3

BDF1 mice received i.v. 1.75×10^6 cpm of $N^{125}IP_4\text{-}PVA_{14}$ (estimated to be ca. 2 μg) mixed with 100 μg of cold $NP_4\text{-}PVA_{14}$. Starting one week before the experiment the mice were given water containing KI (2 g/l) throughout the experiment. After different time intervals the mice were bled and then sacrificed, and their organs were subjected to radioactive counting

Discussion

It was previously reported that a number of hapten-PVA (67,90,92) and DNP-DGL (24) induced hapten-specific unresponsiveness. However, the distribution and the fate of these conjugates after injection into the hosts had not been established. Obviously, the knowledge of the distribution and the kinetics of clearance is of great importance for any conjugates which may be of potential clinical use. For most tolerogens, an important requirement for the induction and maintenance of prolonged and effective tolerance would be their persistence in the host for extended periods. From the results of above experiments, it was concluded that although the concentration of the tolerogenic conjugates of TM-PVA or NIP-PVA dropped rapidly in circulation within the first 24 hours, these conjugates remained in the organs of the body for a long time, especially in the liver and kidney. However, even after removal of the above organs, including liver and kidney, the rest of the carcass still contained high radioactivity.

Humphrey (121) investigated the immunological effects of DNP conjugates of a series of polysaccarides such as Ficoll, levan, dextran, hydroxyethyl starch, type III pneumococcal capsular polysaccharide, alginic and hyaluronic acid, and found that (i) conjugates of acidic polysaccharides were markedly more effective as tolerogens than those of uncharged polysaccharides, and (ii) conjugates of high epitope density were particularly tolerogenic. All the radioiodine labelled polysaccharide conjugates were cleared rapidly from circulation but persisted in the body for long periods. Hence, it would appear that hapten-PVA *in vivo* behave similarly to

other hydrophilic polyvalent hapten conjugates.

Humphrey investigated also the cellular distribution of DNP-polysaccharide conjugates in the liver and spleen, and found that some uncharged polysaccharides were localized only in parenchymal but not in Kupffer cells of the liver. On the other hand, in the spleen, the acidic polysaccharides were predominantly localized in red pulp macrophages, and the neutral polysaccharides were detectable exclusively in the marginal zone of the white pulp. He suggested that the preferential retention of these conjugates in certain areas may be related to their effectiveness as tolerogens. It is possible,however, that the different distribution of acidic and neutral polysaccharides in the liver and spleen may be due to their charge or other properties, and may not be directly related to their tolerogenicity.

IV. B cell tolerance and its relationship to Ts cells

Lee *et al* (67,91) showed in their original studies that tolerogenic DNP-PVA conjugates induce hapten-specific Ts cells in normal BDF1 mice. Seven days after injection of DNP-PVA, the spleen cells of the treated mice could suppress the ability of normal recipients to mount a primary anti-DNP response to immunization with DNP-OA. To exclude the carry-over effect, they further demonstrated that treatment of the normal recipients with cyclophosphamide an hour before cell transfer could abolish this transferred suppression. Moreover, in co-transfer experiments, they showed that spleen T cells from DNP-PVA tolerized animals suppressed the ability of DNP-OA primed spleen cells to mount a secondary anti-DNP antibody response in the adoptive cell transfer system. Therefore, they concluded that Ts cells were at least partly responsible for the long-lasting tolerance induced by DNP-OA. Furthermore, they showed that these conjugates induced also specific B cell tolerance. However, the question as to whether the B cell tolerance was the consequence of the "direct" interaction of DNP-PVA with B cells or was the result of activation of Ts cells acting on B cells had not been settled. It may be suggested that the activation of DNP-specific Ts cells to DNP was a normal associated occurrence, since it had been shown that even priming of mice with DNP-OA plus $Al(OH)_3$ led to the appearance of Ts cells and that this phenomenon was kinetically determined (119). However, since so far no evidence was adduced for the participation of Ts cells in the tolerance induced by TM-PVA conjugates, the following experiments was carried out to further investigate the mechanism underlying this

tolerance.

IV.A. Induction of B cell tolerance by TM_7-PVA_{14}

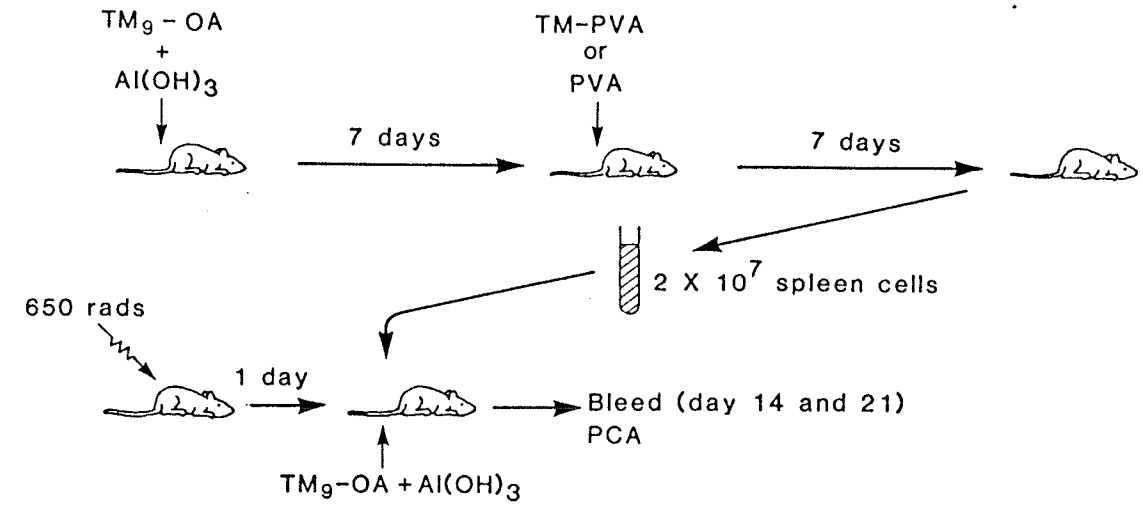
1. The maintenance of the unresponsive state after adoptive cell transfer

In the experiment illustrated in Fig 10 , two groups of BDF1 mice were given a sensitizing dose of TM_9-OA and were treated with either 1 mg TM_7-PVA_{14} or PVA_{14} seven days later. After a further seven-day interval, they were sacrificed and their spleen cells were transferred into two groups of irradiated syngeneic recipients, which were immunized 1 day later with a sensitizing dose of TM_9-OA; the IgE titers of these two groups were determined 14 and 21 days thereafter.

As is evident from the results listed in Fig 10 , the mice which received spleen cells from donors, which had been treated with TM_7-PVA_{14}, showed a markedly depressed anti-TM IgE response (about 90% and 95% suppression on day 14 and 21, respectively) by comparison with recipients of cells from mice which had been treated with PVA_{14} alone. By contrast, the anti-OA IgE response in the recipients was essentially unaffected by treatment of the donor mice with TM_7-PVA_{14}. These results clearly demonstrate that the specific tolerance of the cells to TM from suppressed donors was maintained for at least 21 days in the recipients in spite of the second sensitizing injection.

Furthermore, the tolerant state of the spleen cells induced *in vivo* by TM_7-PVA_{14} persisted after transfer into irradiated mice,

SUPPRESSION OF THE ANTI-TM IgE RESPONSE AFTER ADOPTIVE TRANSFER OF SPLEEN CELLS



Treatment of Donor	Day after immunization of recipient	IgE TITER (PCA) Anti-TM (40 80 160 320 640 1280 2560 5120 10240)	IgE TITER (PCA) Anti-OA (40 80 160 640 1280 2560 5120 10240)
TM-PVA	14		
PVA	14		
TM-PVA	21		
PVA	21		

Fig 10. Suppression of the anti-TM IgE response after adoptive transfer of spleen cells. Two groups of 4 BDF1 mice each were immunized with a sensitizing dose of TM_9-OA. After seven days the test group received an i.p. injection of 1 mg of TM_7-PVA_{14} and the control group 1 mg of PVA_{14}. After another seven days, these mice were sacrificed and their spleen cells were transferred into sublethally irradiated BDF1 mice, which were subsequently immunized with a sensitizing dose of TM_9-OA and the PCA titres of anti-OA and anti-TM of the recipients were measured 14 and 21 days later.

even when the cells had been first cultured *in vitro* in tolerogen-free medium for 36 hours and thoroughly washed prior to cell transfer (Fig 11). In contrast, tolerance induced *in vivo* by TM_7-RNase was easily reversed by this *in vitro* incubation treatment (Fig 11). In this connection, it ought to be noted that in previous experiments (Fig 10), it had been shown that treatment with TM_7-RNase or TM_{21}-BSA resulted in only a transient tolerance, i. e. , treatment with these polyhaptenated proteins resulted in some reduction of the primary anti-TM IgE response, but in no reduction of the secondary response. On the other hand, the tolerance induced by TM-PVA persisted even after booster immunization.

As is evident from the protocol and results illustrated in Fig 12, TM_7-PVA_{14} was also able to tolerize *in vitro* spleen cells from mice which had received 14 days earlier a sensitizing dose of TM_9-OA. For these experiments, spleen cells from immunized mice were incubated (at 5×10^6 cells/ml) in MEM containing TM_7-PVA_{14} (100 μg/ml) or MEM alone for 6 hours. The cells were thoroughly washed and transferred (2×10^7) into sublethally irradiated recipients which were then immunized with a sensitizing dose of TM_9-OA. The results plotted in Fig 12 demonstrate that *in vitro* incubation of TM-primed spleen cells with TM_7-PVA_{14} for as little as 6 hours could markedly inhibit their secondary anti-TM IgE antibody response after transfer to irradiated recipients.

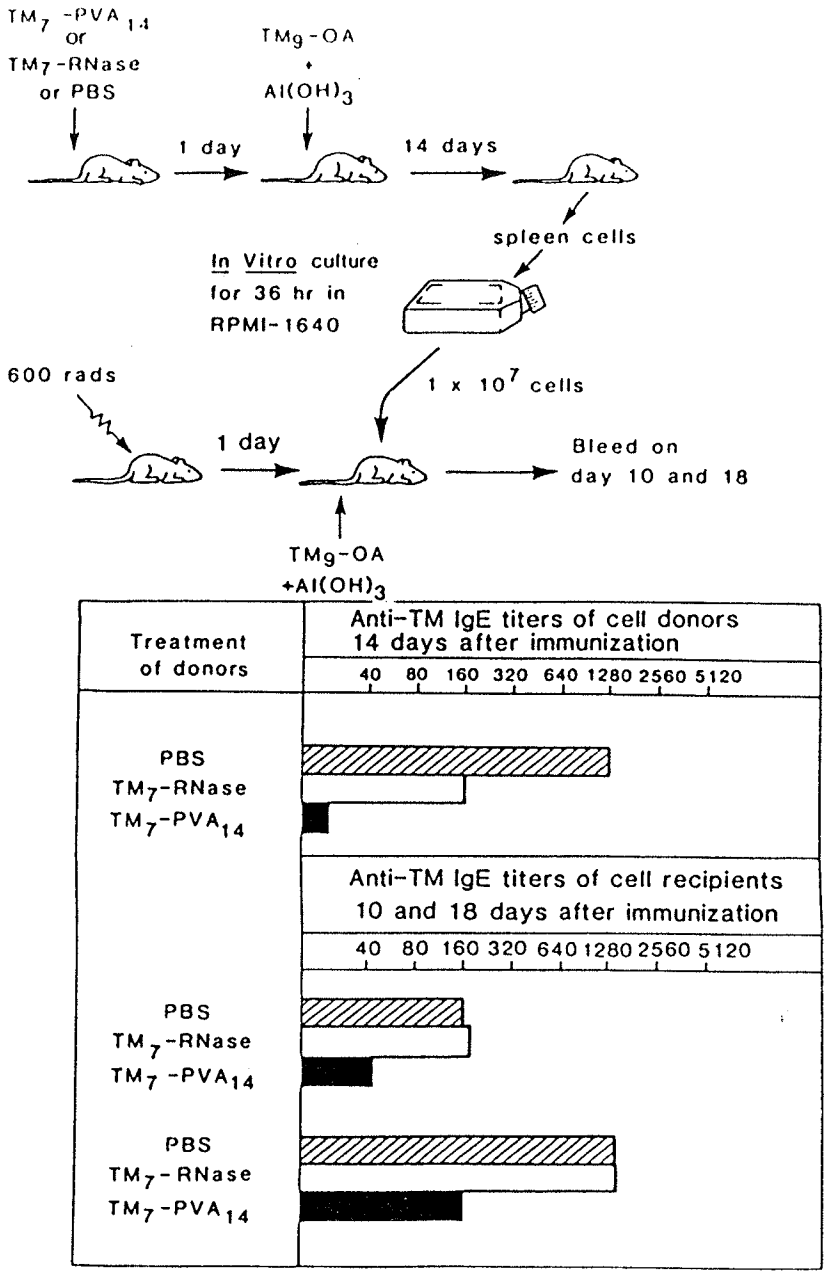


Fig 11. Non-reversible tolerance induced by TM_7-PVA_{14}. Three groups of BDF1 mice were i.p. injected 1 mg of TM_7-PVA_{14} or TM_7-RNase or PBS. After one day, they were immunized with a sensitizing dose of TM_9-OA. Fourteen days after immunization these mice were bled for PCA determination and sacrificed for the collection of their spleen cells. Their spleen cells were incubated *in vitro* in RPMI-1640 for 36 hours, and then transferred, respectively, into sublethally (600 R) irradiated BDF1 mice. These recipients were immediately immunized with a sensitizing dose of TM_9-OA and bled on days 10 and 18 for PCA determinations.

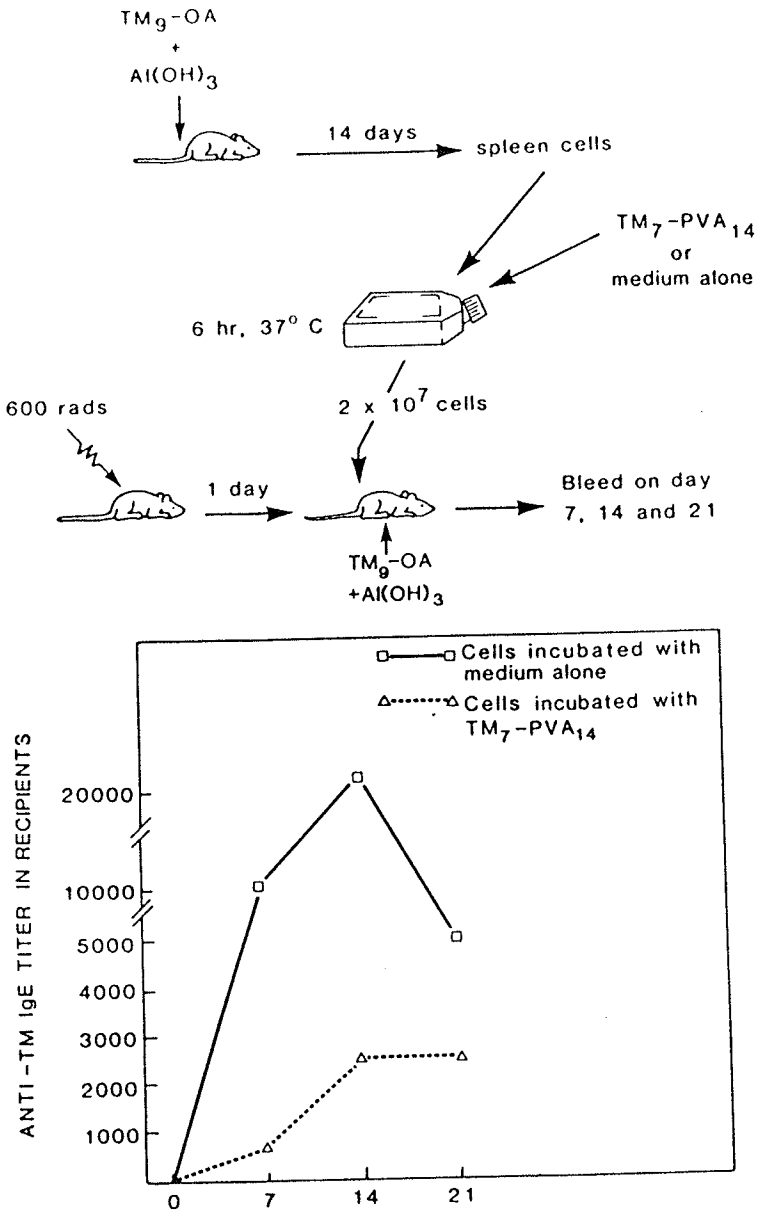


Fig 12. The *in vitro* tolerance of TM-primed spleen cells by TM_7-PVA_{14}. BDF1 mice were immunized with a sensitizing dose of TM_9-OA and after 14 days their spleen cells were collected and incubated in vitro with TM_7-PVA_{14} (100 μg/ml) or medium alone for 6 hours. Then the cells were washed 3 times with MEM and transferred into sublethally irradiated recipients which were then immunized with a sensitizing dose of TM_9-OA. The recipients were bled on days 7, 14, and 21 after immunization for the determination of anti-TM IgE (PCA) titres.

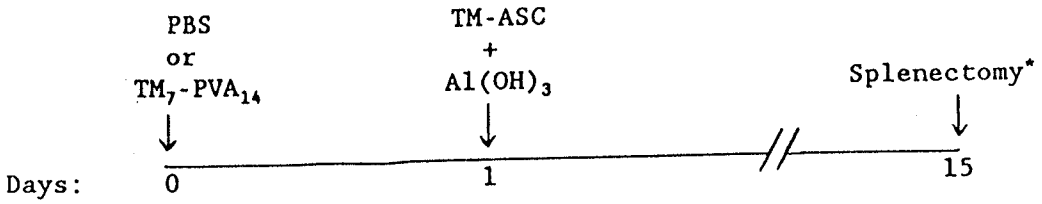
2. Unresponsiveness of spleen cells from tolerized mice on transfer to carrier-primed irradiated recipients.

In this cell transfer experiment (Table 21), OA-primed Th cells, expected to be resistant to 650 Rads (24), were induced in several recipients by a sensitizing dose of OA 28 days prior to irradiation; control mice received PBS in lieu of OA. The cell donors were first treated with PBS or TM_7-PVA_{14}, and on the following day both groups of mice received a sensitizing dose of TM-Asc. Fourteen days after immunization their spleen cells, serving as a source of TM-primed B cells (Sp) or of TM-tolerized B cells (St), respectively, were transferred into the OA-primed, irradiated recipients. A control group of recipients was given normal spleen cells (Sn). The cell recipients were then immediately given a sensitizing dose of TM_9-OA and their IgE titers were determined 7 and 14 days later. From the results listed in Table 21, it is evident that transfer of spleen cells from TM_7-PVA_{14} treated mice into carrier-primed irradiated mice (Group 2) resulted in only a very low IgE titer in relation to that of "control" (Group 1), i.e., the IgE titers of the recipients of the tolerized cells were 6% and 12.5%, respectively, of the titers on day 7 and 14 with respect to the titers of the recipients of primed cells. In essence, the capacity of the tolerized spleen cells to mount an anti-TM IgE response on stimulation with TM_9-OA was reduced to that of normal spleen cells from naive animals (Group 3). In a further control, the recipients (Group 4) were given only PBS prior to irradiation, i.e., in these mice no OA-specific Th cells had been elicited before irradiation; therefore, as expected, these mice mounted only a minimal or no

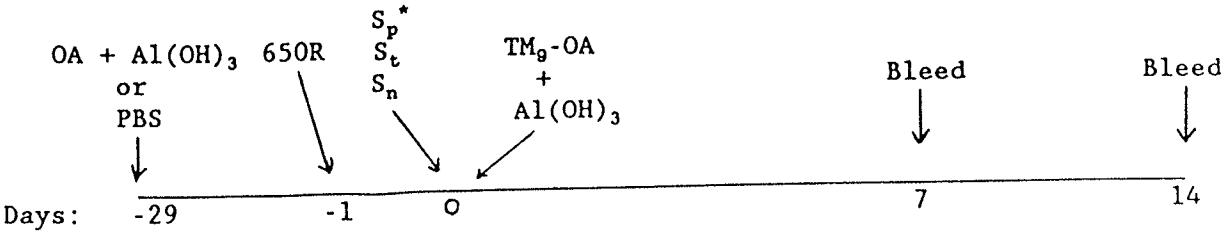
TABLE 21

UNRESPONSIVENESS OF SPLEEN CELLS FROM TOLERIZED MICE ON TRANSFER TO CARRIER PRIMED RECIPIENTS

Treatment of spleen cell donors



Treatment of cell recipients



Treatment of Recipients** (day -29)	Transferred Cells*	IgE titers in recipients			
		Day 7		Day 14	
		anti-TM	anti-OA	anti-TM	anti-OA
1. OA + $Al(OH)_3$	S_p	640	640	>10240	>10240
2. OA + $Al(OH)_3$	S_t	40	640	1280	>10240
3. OA + $Al(OH)_3$	S_n	<40	2560	640	>10240
4. PBS	S_n	<40	<40	<40	<40

* S_p and S_t refer to spleen cells from mice which had been injected with PBS or TM_7-PVA_{14}, respectively, one day before sensitization with TM-ASC in $Al(OH)_3$. Anti-TM IgE titers of donors of S_p and S_t cells were 1280 and 40, respectively.

S_n refers to spleen cells from normal mice.

** Recipients were injected with OA in $Al(OH)_3$ or with PBS. They were then irradiated and one day later were given the cells and a sensitizing dose of TM_9-OA in $Al(OH)_3$; their IgE titers were determined 7 and 14 days later.

response at all to TM and OA after receiving normal spleen cells.

3. Unresponsiveness of splenic B cells from tolerized mice in the presence of carrier primed Th cells

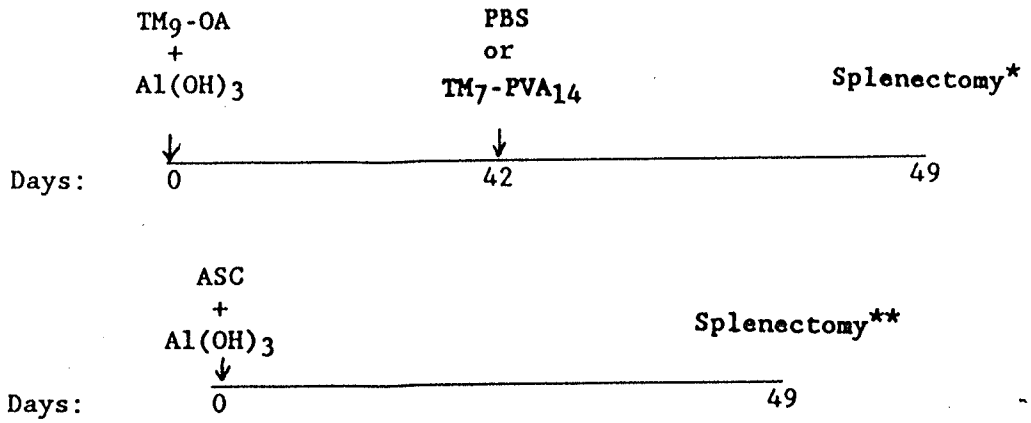
The different types of splenic B cells were obtained as illustrated in the protocol in Table 22. Two groups of donor mice were immunized with a sensitizing dose of TM_9-OA 6 weeks before receiving an injection of 1 mg of TM_7-PVA_{14} or PBS. One week later, their spleen cells were collected and treated with anti-Thy 1.2 plus complement. The residual viable cells were designated as primed B (Bp) or tolerized B (Bt) cells. A third group of normal B (Bn) cells were obtained by treating the normal spleen cells of naive mice with anti-Thy 1.2 plus complement. Spleen cells, as a source of carrier (Asc)-specific Th (Sasc), were harvested from another group of mice immunized 7 weeks previously with 10 μg of Asc in 1 mg of $Al(OH)_3$.

In this experiment BDF1 recipients were given 650 Rads whole body irradiation 24 hours prior to adoptive cell transfer. After transfer of different combinations of cells, the recipients were stimulated with 1 μg of TM-Asc in 1 mg $Al(OH)_3$ and their IgE titers were measured 14 and 21 days later. From the results shown in Table 22, it is evident that the recipients which had received Bp or Bt cells alone did not mount an anti-TM IgE response and that mice which had received Bt with Sasc cells showed a considerably lower anti-TM IgE response than control mice which had been given Bp and Sasc cells, i.e., a reduction of the order of eight fold. To determine if Bt cells had any effect on the cooperation between Bp and Ssac cells, a

TABLE 22

UNRESPONSIVENESS OF SPLENIC B CELLS FROM TOLERIZED MICE IN THE PRESENCE OF CARRIER-PRIMED SPLEEN CELLS.

Treatment of spleen cell donors



Transferred cells	Number	Anti-TM IgE titers in recipients Day 14	Day 21
1. $B_p + S_{asc}$	$10^7 + 10^7$	5120	5120
2. $B_t + S_{asc}$	$10^7 + 10^7$	640	640
3. B_p	$2x10^7$	<40	<40
4. B_t	$2x10^7$	<40	<40
5. S_{asc}	$2x10^7$	640	2560
6. $B_p + S_{asc} + B_n$**	$10^7 + 10^7 + 10^7$	2560	1600
7. $B_p + S_{asc} + B_t$	$10^7 + 10^7 + 10^7$	1280	1400

* B_p and B_t refer to spleen cells treated with anti-Thyl.2 + C which were obtained from mice which were given a sensitizing dose of TM_9-OA on day 0 and subsequently received PBS or TM_7-PVA_{14}, respectively.

B_n refers to spleen cells treated with anti-Thy 1.2 + C which were obtained from normal mice.

** S_{acs} refers to spleen cells obtained from mice which were primed with 10 μg ASC in $Al(OH)_3$ 49 days earlier.

*** All recipients were given the cells and a sensitizing dose of TM_9-ASC in $Al(OH)_3$ one day after irradiation (650 R); their IgE titres were determined 14 and 21 days later.

mixture of Bp and Sasc cells with either Bn or Bt cells was injected into the recipient mice (Group 6 and 7, respectively); no difference was evident in subsequent IgE responses of these two groups of mice.

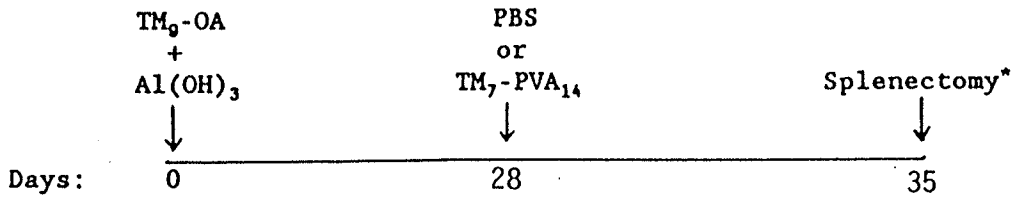
All these results taken together suggested that B cells were tolerized by treatment with TM_7-PVA_{14} and that Bt cells had no suppressive effect on the cooperation between Bp and Th cells. However, the above results may be also interpreted by postulating that (i) small tolerogenic doses of TM_7-PVA_{14} were carried over from the original donors by B cells or other spleen cells, such as macrophages, and/or (ii) treatment with TM_7-PVA_{14} induced a population of hapten specific Ts cells which were not completely eliminated by treatment with anti-Thy 1.2 plus complement. With regard to the latter possibility, it is to be noted that splenic T cells have been reported to possess lower density of Thy 1.2 antigen on their surfaces than thymocytes (122,24) and, in our hands, the monoclonal anti-Thy 1.2 preparation plus complement did not eliminate all the T cell activity in some experiments. However, both possibilities were virtually excluded by the following experiment.

For this experiment (Table 23), Bp and Bt cells were obtained from mice which had been first primed with TM_9-OA and then treated with PBS or TM_7-PVA_{14}, respectively, and the carrier (Asc) primed Th cells (Tasc) were obtained from nylon wool purified T cells of the spleens of mice which had been immunized 6 weeks before with 10 μg of Asc in $Al(OH)_3$. Other groups of donors were treated one week before sacrifice with TM_7-PVA_{14} or PBS: their spleen cells were designated as St or Sn cells. All recipients of cells received a sensitizing dose of 1 μg

TABLE 23

SPLEEN CELLS FROM TOLERIZED DONORS DO NOT AFFECT ACTIVITY OF PRIMED B CELLS IN TRANSFER EXPERIMENTS

Treatment of spleen cell donors



Transferred cells	Cell number	IgE titers (anti-TM) in recipients**** Day 14	Day 21
1. $B_p + T_{ASC}$	$10^7 + 10^7$	2820	1280
2. $B_t + T_{ASC}$	$10^7 + 10^7$	270	270
3. $B_p + T_{ASC} + S_t$	$10^7 + 10^7 + 2x10^7$	640	200
4. $B_p + T_{ASC} + S_n$	$10^7 + 10^7 + 2x10^7$	810	200

* B_p and B_t refer to spleen cells treated with anti-Thy1.2 + C, which were obtained from mice which were given a sensitizing dose of TM_9-OA on day and subsequently treated with PBS or TM_7-PVA_{14} respectively.

** T_{ASC} refers to splenic T cells (nylon wool purified) obtained from mice prime with ASC.

*** S_n and S_t refer to spleen cells from mice injected with PBS or TM_7-PVA.

**** All recipients received the cells and a sensitizing dose of 10 μg TM_9-ASC in $Al(OH)_3$ one day after irradiation (600 R); their IgE titers were determined 14 an days later.

TM-Asc immediately after cell transfer. The response of mice which had received Bt + Tasc cells (Group 2)) was much weaker (i.e., 10 to 20%) than the response of mice which had received Bp + Tasc cells (Group 1), again confirming that B cells had been tolerized by treatment of the donors with TM_7-PVA_{14}. Moreover, the co-operation between Bp and Tasc cells was not affected by either Sn or St cells; this result added further support to the conclusions that (i) tolerogenic amounts of TM_7-PVA_{14} were not carried over by spleen cells of the donors to the recipients, and (ii) there was no demonstrable Ts cell activity one week after treatment with TM_7-PVA_{14}. Further support for the lack of participation of Ts cells in the tolerance induced by TM_7-PVA_{14} was provided in the following series of experiments.

IV.B. The role of Ts cells in the immune tolerance induced by TM_7-PVA_{14}

This series of experiments was designed to demonstrate the possible involvement of Ts cells in the tolerance induced by TM-PVA conjugates and to investigate the relationship between the reduction of the anti-TM IgE response and the putative Ts cells.

1. Failure of spleen cells from tolerized mice to suppress the anti-TM IgE response of normal mice on immunization with TM_9-OA.

Donor BDF1 mice were injected i.p. with 1 mg of TM_7-PVA_{14} or PBS and 1 week later these two groups were sacrificed; 3×10^7 or 6×10^7 of their spleen cells were transferred i.v. into syngeneic animals. The cell recipients were given a sensitizing dose of TM_9-OA immediately after cell transfer and the anti-TM and anti-OA IgE titers were measured on days 14 and 21. The results of two such experiments

demonstrated that both the anti-TM and anti-OA IgE titers of mice which had received spleen cells from TM_7-PVA_{14} treated donors were not significantly different from the control recipients which recived normal spleen cells.

In two similar experiments the cell donors were sacrificed respectively 2 and 3 weeks after injection of 1 mg of TM_7-PVA_{14} or PBS. After cell transfer the syngeneic recipients were given a sensitizing dose of TM_9-OA. Again, as in the earlier experiment, there was no indication that TM_7-PVA_{14} had induced any transferable Ts cells , in spite of the fact that the donors exhibited a long-lasting anti-TM tolerance.

Since the failure to demonstrate Ts cells in BDF1 mice might be attributable to these mice representing a strong IgE responder strain and that hence the Ts cell activity may be overshadowed by the much stronger Th cell activity, some experiments were performed in C57BL/6 mice which give a moderate IgE response. For this purpose, two injections of 1 mg TM_7-PVA_{14} each were given one day apart to groups of C57BL/6 mice. Seven days after the second injection, the spleen cells were transferred into normal C57BL/6 recipients which were subsequently immunized with 10 μg of TM_9-OA in 1 mg $Al(OH)_3$. Although TM-PVA was found to induce unresponsiveness to TM in C57BL/6 mice, the transfer of 2.5×10^7 cells from immunosuppressed mice failed to reveal any significant Ts cell activity in the recipients.

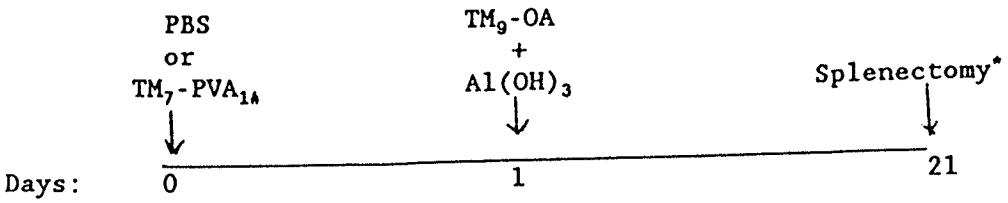
2. Failure of spleen cells from tolerized mice to suppress the response of spleen cells from primed mice after transfer to irradiated recipients

To explore the possibility that the presence of TM-specific Ts cells, induced by administration of TM-PVA conjugates, could be demonstrated in adoptive cell transfer experiments, BDF1 mice were given either PBS or 1 mg of TM_7-PVA_{14} 1 day before immunization with a sensitizng dose of TM_9-OA. After 20 days, these mice were bled, sacrificed and their spleen cells designated (as previously) as Sp and St cells, respectively. The Sp and St cells were transferred separately or together into sublethally irradiated syngeneic animals which were given a sensitizing dose of TM_9-OA. If the St cells from TM_7-PVA_{14} treated mice contained Ts cells, they should have suppressed the response of the Sp cells to TM_9-OA. As a control, the same number (2×10^7) of Sn cells of naive BDF1 mice were substituted for St cells and the mixed population was transferred into irradiated syngeneic mice. As is evident from the data listed in Table 24, the tolerized spleen cells of the donor mice (Group 2) which had been treated with TM_7-PVA_{14} remained suppressed after adoptive transfer in spite of the recipients being given an additional sensitizing dose of TM_9-OA; by contrast the recipients of Sp cells (Group 1) produced anti-TM IgE antibodies in high titers. Moreover, IgE titers of recipients of mixture of Sp and St cells (Group 3) did not differ significantly from the titers of recipients of Sp and Sn cells (Group 4). In additional experiments (data not shown), the donor mice were treated with TM_7-PVA_{14} or PVA_{14} seven days after sensitization using a similar

TABLE 24

CO-TRANSFER OF SPLEEN CELLS FROM TOLERIZED AND PRIMED MICE

Treatment of spleen cell donors



Transferred Cells*	IgE titers of recipients on day 14** anti-TM	anti-OA
1. $2x10^7$ S_p	4310	5120
2. $2x10^7$ S_t	670	6170
3. $2x10^7$ S_p + $2x10^7$ S_t	2560	6270
4. $2x10^7$ S_p + $2x10^7$ S_n***	3930	3800

* S_p and S_t refer to spleen cells from mice which were injected with PBS or TM_7-PVA_{14}, respectively, one day before sensitization with TM_9-OA in $Al(OH)_3$. The anti-TM IgE titers of the donors of S_p and S_t were 2920 and 70 respectively; the corresponding anti-OA titers were 2690 and 2920.

** All recipients were given the cells and a sensitizing dose of TM_9-OA in $Al(OH)_3$ one day after irradiation; their IgE titers were determined 14 and 21 days later.

*** S_n refers to spleen cells from normal mice.

transfer protocol to that illustrated in Table 24; again no active suppression was demonstrated on cell transfer, although the donor mice treated with TM_7-PVA_{14} had significantly lower PCA titres than those treated with PVA_{14}.

In the above experiments, the attempts to induce St cells involved mice which had received, in addition to an injection of TM_7-PVA_{14}, a sensitizing dose of TM_9-OA. Hence, this protocol may not have favoured the detection of specific Ts cells, particularly if the Ts cell activity were relatively weak and may have, therefore, been overwhelmed and masked by the Th cell activity. Hence, in the experiment illustrated in Table 25, the donor mice were given 1 mg of TM_7-PVA_{14} or PBS in the absence of a sensitizing dose of TM_9-OA. One week later these mice were sacrificed and their spleen cells were separately mixed with spleen cells of a third donor group which had been immunized 40 days earlier with a sensitizing dose of TM_9-OA and which served as a source of primed cells. The mixed cell populations were transferred into sublethally irradiated BDF1 mice and stimulated with a sensitizing dose of TM_9-OA. As is evident from the results in Table 25, there was no difference in the antibody responses of recipients of Sp cells in the presence of either St or Sn cells.

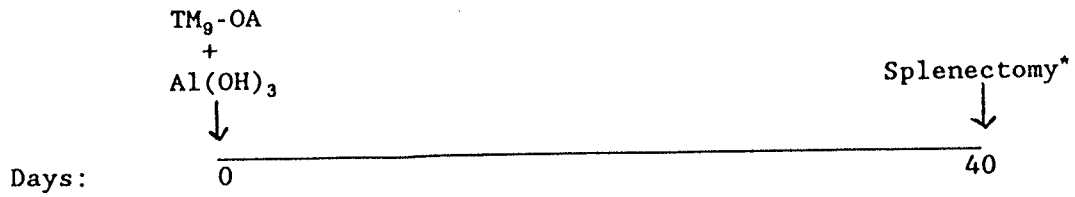
3. <u>B cell tolerance may not be the consequence of activation of Ts cells</u>

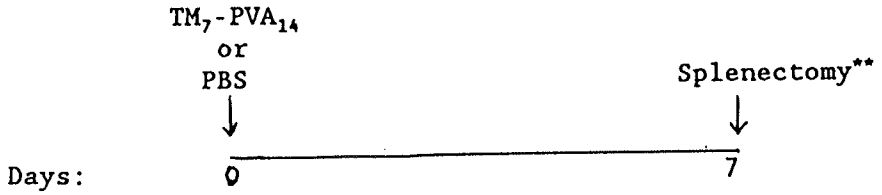
Manipulation of the unresponsive animals with low dose of irradiation or with cyclophosphamide was deemed to provide another approach to probe the relationship between Ts cells and the unresponsiveness induced by TM_7-PVA_{14}, since several authors reported

TABLE 25

CO-TRANSFER OF SPLEEN CELLS FROM TOLERIZED AND PRIMED MICE

Treatment of spleen cell donors





Transferred cells* Group	Number	IgE anti-TM titer of recipients** day 7	day 14
S_p	$1X10^7$	2820	11 240
S_t	$1x10^7$	<10	<10
S_n	$1x10^7$	<10	<10
$S_p + S_n$	$1x10^7 + 1x10^7$	2560	9870
$S_p + S_t$	$1x10^7 + 1x10^7$	1710	11 240
$S_p + S_n$	$1x10^7 + 4x10^7$	1280	5720
$s_p + S_t$	$1x10^7 + 4x10^7$	810	5120

* S_p refers to spleen cells from mice which had received a sensitizing dose of TM_9-OA in $Al(OH)_3$.

** S_t and S_n refer to spleen cells from mice which were injected with TM_7-PVA_{14} or PBS_7, respectively.

*** All recipients were given the cells and a sensitizing dose of TM_9-OA in $Al(OH)_3$ one day after irradiation; their IgE titers were determined 7 and 14 days later.

that low dose irradiation (7,36,37) or treatment with cyclophosphamide (37,75) could prevent the development of Ts cells.

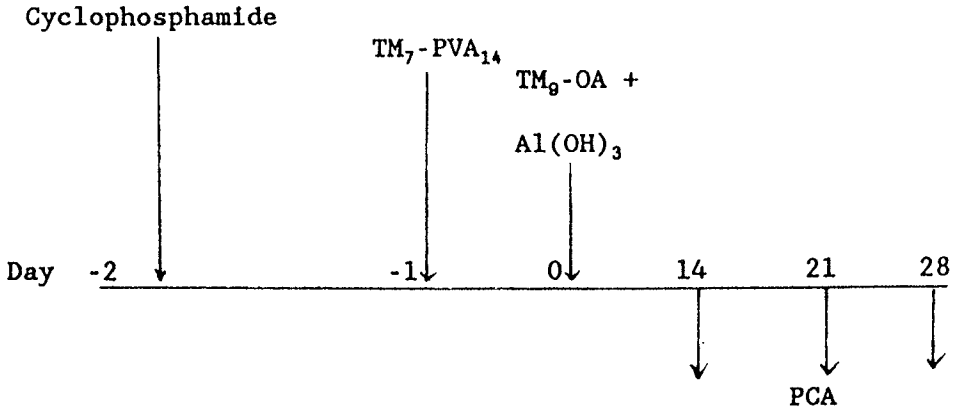
To investigate the above relationship, BDF1 mice were given an i.p. dose of 0.6 mg cyclophosphamide in 0.3 ml (approximately 20 mg/kg). After one day the mice were injected with 1 mg of TM_7-PVA_{14} and on a subsequent day they were immunized with a sensitizing dose of TM_9-OA; their sera were collected 14, 21 and 28 days after immunization for determination of PCA titers. From Table 26, it may be concluded that cyclophosphamide treatment did not have any significant influence on the IgE antibody response. Thus, from a comparison of PCA titers of Groups I and Group II, cyclophosphamide was shown not to have enhancing effect on the IgE antibody response in BDF1 mice. However, it did not abolish or diminish the unresponsiveness induced by TM_7-PVA_{14}, i.e., the B cell tolerance persisted despite the potential of cyclophosphamide to delete Ts cells.

Using a similar protocol with the only difference that dose of irradiation (250 - 300 R) was substituted for cyclophosphamide either one day before or seven days after the treatment of TM_7-PVA_{14}, it was shown that low dose irradiation did not abolish the suppressive effect induced by TM_7-PVA_{14}. It may be, therefore, concluded that neither cyclophosphamide nor low dose irrdiation could reverse the unresponsive state induced by TM_7-PVA_{14}.

4. Demonstration of Ts cells in the DNP-PVA system

To confirm or refute the original findings in this Laboratory that other hapten-PVA conjugates could indeed induce Ts cells, 1 mg of each of two different DNP-PVA preparations, i.e., DNP_2-PVA_3 and DNP_2-PVA_{14} ,

Table 26. The influence of cyclophosphamide on the tolerogenic effect of TM_7-PVA_{14}



Groups	Treatment on day -2	Treatment on day -1	anti-TM PCA titres day 14	day 21	day 28
I	cyclophos-phamide	TM_7-PVA_{14}	60	80	80
II	PBS	TM_7-PVA_{14}	40	40	40
III	cyclophos-phamide	PBS	890	1,280	1,280
IV	PBS	PBS	1,340	1,340	1,490

Mice were given cyclophosphamide or PBS on day -2 and on the next day, Groups I and II received 1 mg of TM_7-PVA_{14}, whereas Groups III and IV only with PBS. On day 0, all the mice were immunized with a sensitizing dose of TM_9-OA.

was injected into BDF1 mice. After one week, different numbers of their spleen cells (2.5×10^7, 5×10^7, 1×10^8) were transferred into normal syngeneic animals and the recipients were subsequently immunized with DNP_3-OA, and their anti-DNP IgE responses were measured 14 and 21 days thereafter. As illustrated in the results of Table 27, mice which received spleen cells from animals treated with DNP_2-PVA_{14} or DNP_2-PVA_3 gave markedly weaker anti-DNP IgE response than that of the control mice. However, their anti-OA IgE responses did not differ from each other significantly (data not shown).

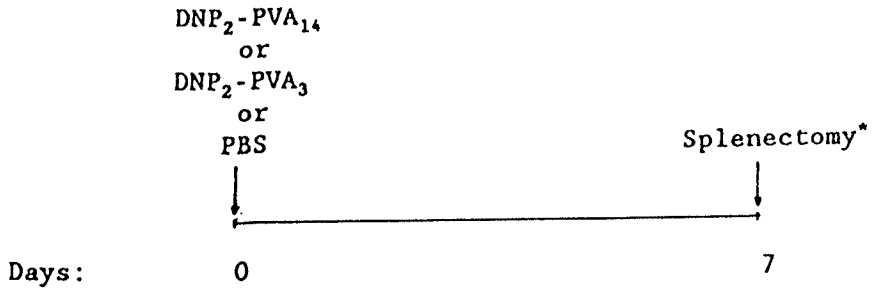
In another experiment, using a similar protocol to that shown in Table 25 and reference (91), BDF1 mice were injected with 1 mg of DNP_2-PVA_3 or PBS and their spleen cells were designated as St and Sn cells, respectively. Spleen cells from DNP-primed mice (Sp cells) were obtained from a third group of donor mice which had been immunized 3 weeks prior to the cell transfer with a sensitizing dose of DNP_3-OA. These cells were transferred either alone, or in combination with St or Sn cells, into sublethally irradiated syngeneic recipients which were immediately immunized with a sensitizing dose of DNP_3-OA. Mice which had received 1×10^7 Sp plus 4×10^7 St cells had a 2 to 4 fold reduction in their anti-TM IgE response as compared to the group which had received 1×10^7 Sp plus 4×10^7 Sn cells (data not shown); hence, this finding is consistent with the earlier published data supporting that the spleen cells of mice receiving DNP-PVA conjugates contained Ts cells, which had the capacity to overwhelm the ability of DNP-primed spleen cells to respond to booster immunization with DNP_3-OA.

It may be argued that the suppressive effect illustrated in Table 27 was actually due to the carry-over of the tolerogenic amounts

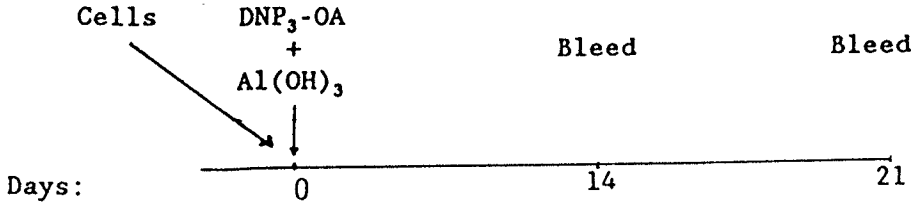
TABLE 27

ABILITY OF DNP-PVA TO INDUCE TRANSFERABLE SUPPRESSION.

Treatment of spleen cell donors



Treatment of cell recipients



Treatment of Donors	Number of Transferred cells	IgE titres (anti-DNP) in recipients Day 14	Day 21
PBS	2.5×10^7	2560	450
DNP_2-PVA_{14}	2.5×10^7	810	150
DNP_2-PVA_3	2.5×10^7	480	100
PBS	5×10^7	1410	260
DNP_2-PVA_{14}	5×10^7	310	70
DNP_2-PVA_3	5×10^7	450	70
PBS	1×10^8	1410	260
DNP_2-PVA_{14}	1×10^8	570	80
DNP_2-PVA_3	1×10^8	290	80

* Spleen cells from mice treated with DNP_2-PVA_{14}, DNP_2-PVA_3 or PBS were transferred into normal (non-irradiated) mice immediately before a sensitizing injection of DNP_3-OA in $Al(OH)_3$.

of the DNP-PVA conjugates along with the transferred putative Ts cells. However, this possiblity was virtually excluded by the experiments previously carried out in this Department (91) by the treatment of the recipients of these cells with cyclophosphamide .

Discussion

The results of the above experiments demonstrate that TM-PVA conjugates induced hapten-specific tolerance in BDF1 mice that was maintained after transfer of spleen cells of immunosuppressed donors into irradiated mice in spite of the subsequent immunization of the latter with a sensitizing dose of TM_9-OA in $Al(OH)_3$. This tolerant state was not reversed by *in vitro* culture of the immunosuppressed spleen cells (in the absence of tolerogen) for 36 hours and it is important to note that even primed cells could be tolerized *in vitro* by culture with TM_7-PVA_{14}. These findings are consistent with the interpretation that TM_7-PVA_{14} conjugates induced B cell tolerance either by inactivation of B cells or by the blockade of their receptors by the multivalent tolerogenic conjugates. Since unfractionated spleen cells were used in the first experiments of this chapter, the observed unresponsiveness could also have been attributed to other mechanisms, such as tolerization of Th cells or induction of Ts cells. However, the results of subsequent cell transfer experiments utilizing different combinations of cells confirmed that TM_7-PVA_{14} induced B cell tolerance, since splenic B cells from tolerized mice failed to interact with either Th cells of carrier-primed intact recipients, or in adoptive transfer experiments with Th cells derived from carrier-primed donors.

Furthermore, the observed tolerance did not appear to be due to either the carry-over of tolerogen with the transferred cells nor to Ts cells, since spleen cells from tolerized donors failed to suppress the interaction between TM-primed B cells and carrier-primed Th cells. On the other hand, the results of the earlier studies in this Laboratory supported the conclusion that in addition to B cell tolerance, DNP_2-PVA_{14} induced also demonstrable Ts cells. Indeed, the activation of Ts cells by DNP-PVA conjugates was confirmed also in the present study, just for the sake of establishing or refuting the validity of the conclusion of the earlier studies (91). Hence it would appear that the participation of Ts cells in the suppression induced by hapten-PVA conjugates may not be a uniform mechanism for all hapten systems. Since, in spite of a great deal of effort, no evidence was obtained in the present study that TM-PVA induced TM-specific Ts cells, it may be suggested that the induction of Ts cells by some hapten-PVA conjugates, and not by others depends on the nature of the particular haptenic group. Nevertheless, it may be stated that all hapten-PVA conjugates, irrespective of their haptenic residues, induce tolerance of B cells.

In this connection, it ought to be pointed out that whereas the previously used PVA conjugates consisted of uncharged haptens, the TM-PVA conjugates were acidic, i.e., contained a free carboxyl group for each haptenic residue. Hence, future studies with PVA conjugates of haptenic molecules differing only in their charges, i.e., with electrically neutral, or positively, or negatively charged haptenic residues, may be warranted to establish the plausibility of this suggestion.

GENERAL DISCUSSION

The results of the present study support the conclusion that administration of PVA conjugates of TM suppressed the induction of anti-TM antibodies of the IgE and other immunoglobulin classes. Moreover, and even more importantly, with respect to their potential therapeutic use in allergic conditions caused by industrial chemicals, these conjugates suppressed the ongoing anti-TM antibody responses in presensitized mice. In conformity with the findings of previous studies in this Laboratory (91,92) which demonstrated that BPO-PVA and DNP-PVA specifically suppressed the anti-BPO and anti-DNP IgE responses, TM-PVA suppressed exclusively the anti-hapten response without affecting the anti-carrier response.

It was noted that conjugates with an average epitope density as low as one were capable of suppressing anti-TM responses to immunization with TM_3-OA. However, when the mice were immunized with TM_9-OA, i.e., an immunogen with a higher epitope density which induced a stronger anti-TM response, it was shown that the degree of suppressogenicity of the TM-PVA conjugates increased with the hapten valency of the conjugates and their epitope density. Thus, the conjugate TM_7-PVA_{14} which was the most tolerogenic had the highest hapten valency and the highest epitope density per unit PVA chain length among the conjugates tested.

It was somewhat surprising to find that the conjugates containing the hexanediamine spacer did not appear to be more tolerogenic than the conjugates with the TM groups directly coupled onto the PVA backbone, since one would expect that the TM residues of the former conjugates

would be more readily accessible for the interaction with anti-TM receptors on the appropriate B and T cells which are involved in the anti-TM response. However, this may be primarily a reflection of the fact that the PVA conjugates synthesized by coupling the TM groups directly onto the PVA molecules had a higher epitope density. Moreover, as implied above, it appears that a conjugate consisting of a large number of TM groups on a longer PVA molecule, e.g., an average of seven TM groups per PVA molecule, with a molecular weight of 14,000, possessed a markedly higher tolerogenic capacity than TM-PVA conjugates of lower epitope density. In this connection is is to be noted that Liu *et al* (123) demonstrated that administration of their highly substituted conjugate of TMA with DGL of molecular weight of 63,700 (TM_{36}-DGL) completely suppressed the anti-TM IgE response and reduced substantially the levels of anti-TM antibodies belonging to other classes. However, these workers did not study the effect of conjugates of lower epitope density, which would be more desirable for therapeutic use in clinical situations.

Like many conjugates of haptens with nonimmunogenic carriers (24,79-82), the tolerance induced by TM-PVA conjugates involved B cell tolerance, as shown by the adoptive cell transfer experiments described above (Table 21 & 22, p. 110 & 112). Thus, tolerance at the B cell level appears to be readily achieved with carriers which are either poorly immunogenic or nonimmunogenic for T cells. In other words, under the conditions not favoring the development of Th cells, direct interaction of the tolerogen at an appropriate concentration with B cell receptors will result in tolerance of B cells.

It was observed that although TM-PVA could dramatically suppress

murine anti-TM IgE response, some low levels of residual IgE antibodies were detected. It has been reported that mature B lymphocytes are relatively resistant to tolerogenic signals (56). Although it was shown that bone marrow cells were sensitive to the tolerogenic TM-PVA conjugate (data not shown), it was difficult to draw the conclusion that immature B cells are more sensitive to the tolerogen, since bone marrow cells themselves consist of cells at different stages of development. It is possible that this aspect could be clarified with the aid of the splenic focusing assay (116), in order to determine the developmental stages when B cells can be tolerized by hapten-PVA conjugates.

It was shown in Table 9 (p.66), that 4 weeks after the injection of 1 mg of TM_7-PVA_{14} , the tolerant state gradually disappeared. It is generally believed that the rate of spontaneous recovery reflects the generation of immunocompetent lymphocytes from the stem cells. Since residual hapten-PVA persisted for more than several weeks, the conjugate may be capable of tolerizing some new cells as the latter emerge from the stem cell pool. However, as the concentration of the tolerogen declines, the animal gradually recovers from the tolerant state.

It was shown in the above experiments (Fig 9, p.85) that radioresistant IgE responses were sensitive to the treatment with TM_7-PVA_{14}, and that antibody secretion by antibody forming cells (either normal PFCs or hybridoma cells) were suppressed by hapten-PVA conjugates. It may be, therefore, suggested that TM-PVA may act on the putative long-lived IgE forming cells by a mechanism of effector cell blockade. However, the basis of long term production of IgE has

as yet not been elucidated. Peeters et al (8,9) had demonstrated, by adoptive transfer, the persistence of the anti-OA IgE response into irradiated syngeneic recipients in the absence of further antigen stimulation. However, in similar experiments the mainteance of anti-DNP and anti-TM IgE responses in adoptive transfer system failed, respectively, in their studies and in the present investigation, despite many attempts. It was noted that the anti-hapten IgE-PFCs in the spleen and lymph nodes (mesenteric, inguinal, and thoracic) dropped to near the background levels 2-3 weeks after immunization with minutes doses of TM_9-OA in $Al(OH)_3$, while the anti-TM antibody titre in the blood persisted for many months. Therefore, the demonstration of the source of these IgE producing cells seems to be critical for the elucidation of persistent IgE responses in future investigations. As discussed in LITERATURE REVIEW, Holt *et al* (11, 13), using a more sensitive method, viz., ELISA-PFC assay, demonstrated that IgE producing cells existed in the bone marrow and draining lymph nodes. However, the present author was not aware of this procedure until the completion of his experiments for the thesis. Hence the possibility still exists that anti-TM IgE PFCs may have escaped detection in the present study because of the possible lower sensitivity of the method used for the enumeration of PFCs.

It was reported by Lee and Sehon (67), that DNP-PVA induced hapten-specific Ts cells, which were at least in part responsible for the tolerance induced by DNP-PVA. The above discovery was confirmed in the experiments as shown in Table 27 (p.124) using DNP_2-PVA_3 and DNP_3-PVA_{14} .

In general, Ts are activated under two circumstances: 1) Ts

cells are induced during a normal immune response. It was reported by Schwenk *et al* (119) of this Laboratory that even with an immunization protocol which results in a persistent IgE response., Ts cells may be demonstrated at a certain period after antigen stimulation. 2) Ts cells can be induced under a variety of conditions that favor the development of Ts over Th cells, such as injecting antigens in soluble form without an adjuvant, the use of low respoonder animals, or intravenous administration of antigens. The generation of Ts cellss to DNP may be a normal ocurrence of the immune response and their presence may be coincidental to the induction of the unresponsive state of B cells or serve as an additional safe-lock mechanism. It was reported by Weigle *et al* (125) that suppressor cells do not play a mojor role in the induction or maintenance of immunologic unresponsiveness to T-dependent protein antigens, such as human gamma globulin, even though Ts activity could be demonstrated at certain times. Their findings led to the following conclusions: 1) tolerance can be established in the absence of detectable suppressor cells, 2) the level of suppressor cell activity does not necessarily correlate with the duration of the unresponsive state.

The major mechanism for induction of unresponsiveness induced by several types of tolerogenic conjugates, such as DNP-DGL, DNP-dextran, and DNP-levan, appears to involve B cell toelerance seemingly in the absence of detectable Ts cells. Similarly, in the present study, although TM-PVA conjugates proved to be good tolerogens, no evidence could be adduced for the participation of hapten-specific Ts cells. Thus, the application of measures commonly used for elimination of Ts cells, such as low dose irradiation or treatment with cyclophosphamide,

did not result in the abrogation of the tolerant state induced by TM-PVA and, hence, as stated above the conclusion was drawn that Ts cell activation was not mandatory for the maintenance of the tolerant state.

The reason for the difference in the ability of TM-PVA and DNP-PVA to induce Ts cells remains obscure. However, as noted earlier, DNP-PVA conjugates with an epitope density of 1 were more effective tolerogens than TM-PVA conjugates of a similar epitope density; in fact TM-PVA conjugates of a higher epitope density, e.g., TM_7-PVA_{14} approached the level of suppressogenicity of DNP_1-PVA_{14}. In relation to this discrepancy, it may be relevant to point out that whereas the DNP_2-PVA_3 and DNP_2-PVA_{14} conjugates are neutral, TM_7-PVA_{14} is acidic due to the carboxyl groups of the haptenic residues. Hence, it may be speculated that the neutral conjugates may interact with the corresponding immunocmpetent cells in such a manner as to favor the induction of a significant number of Ts cells, whereas this mechanism may not be applicable for the acidic TM-PVA conjugates.

One may also suggest that the protocols used in the above experiments were not best suited for the demonstration of any Ts activity induced by TM-PVA. However, in the absence of other known experimental procedure available for the demonstration of Ts cells, the only conslusion which may be offered at this time is that if they had actually been induced, their presence was short-lived and was not mandatory for the maintenance of the tolerant state. Hence, it is concluded that the primary mechanism for the suppressive action of TM-PVA conjugates involved the induction of long lasting B cell tolerance.

The capacity of hapten-PVA conjugates to induce hapten-specific tolerance may represent a valauable characteristic of this class of compounds for the development of therapeutic regimens for the treatment of patients allergic to low molecular weight substances such as industrial chemicals (e.g., TMA, TDI, plicatic acid, phthalic anhydride) or pharmacological drugs (e.g., penicillin). Although the multivalency of these conjugates would prsent some problems in treating sensitized patients, by virtue of their ability to trigger anaphylaxis or a systemic anaphylactoid reaction, it may be noted that some of the hapten-PVA conjugates, even with an average eiptope density of 1, were effective tolergoens (67), i.e., such conjugates did not induce anaphylactic shock in sensitized mice (Table 11, p.72) and were less effective at inducing PCA reactions (Table 10, p.70).

In addition, since hapten-PVA conjugates were shown to suppress also antibodies belonging to other immunoglobulin classes, it may be envisaged that conjugates of PVA and small peptides (e.g., antigenic determinants of a protein) could be used for the treatment of certain autoimmune diseases for which the responsible antigen(s) have been characterized, e.g., immunodominant sites of acetylcholine receptors in the case of myasthenia gravis (126,127).

In summary, hapten-PVA conjugate have proved to be effective tolerogens in a murine model system and it may, therefore, be suggested that they have the potential of being used therapeutically for the treatment of (a) professional allergies induced by chemically highly reactive compounds such as TMA and (b) drug allergies induced by effective pharmacological agents for a variety of diseases which, however, cannot be used liberally because of their inherent

allergencity.

In conclusion, the following findings of this study are considered to represent ORIGINAL CONTRIBUTION TO KNOWLEDGE:

1. A series of TM-PVA or TM-NH-PVA conjugates were synthesized using different chemical methods, and were shown to be effective in dampening anti-TM IgE responses in mice. The tolerogenicity of these types of conjugates was essentially similar.

2. The *in vivo* distribution of these conjugates, was studied both in circulation and in organs, such as liver, kidney, spleen and other lymphoid organs. It was found that although the concentration of hapten-PVA dropped rapidly in circulation ($t_{1/2}$ =1.4 hours for TM_7-PVA_{14}), they were detected in organs, such as liver and kidney for up to 100 days (for NIP_4-PVA_{14}).

3. It was shown that the radioresistant anti-hapten IgE responses, which had been attributed to putative long-lived IgE forming cells, were almost totally abrogated by treatment with TM_7-PVA_{14}. The mechanism for this effect appeared to involve the blockade of antibody forming cells.

4. It was shown that B cell tolerance was the main mechanism of the immune tolerance induced by TM_7-PVA_{14}, in the absence of demonstrable Ts activity, in contrast to the dual tolerogenic effects of DNP-PVA resulting in the switching off B cells and the activation of Ts cells.

REFERENCES

1. Bach MK *et al*: Immediate hypersensitivity: Modern concepts and developments. Marcel Dekker Inc, 1978

2. Sherman WB *et al*: J Allergy 11:225, 1940

3. Austen KF *et al:* Physiology, immunopharmacology and treatment. New York, 1973, Academic Press

4. Levine BB *et al*: Int Arch Allergy 39:156, 1970

5. Vaz EM *et al*: Immunology 21:11, 1971

6. Vaz NM *et al*: J Exp Med 134:1335, 1971

7. Watanabe N *et al*: J Exp Med 143:833, 1976

8. Peeters S *et al* Immunol 121:1596, 1978

9. Peeters S *et al*: J Immunol 43:25, 1981

10 Okudaira H *et al*: Cell Immunol 58:188, 1983

11. Holt PG *et al*: Int Arch Allergy appl Immunol 71:188, 1983

12. Sedgwick JD *et al* Int Arch Allergy appl Immun 65:162, 1981

13. Holt PG *et al*: Int Arch Allergy appl Immun 77:45, 1985

14. Okudaira H *et al*: Int Arch Allergy appl Immun 65:162, 1981

15. Warner NL: Adv Immunol 19:67, 1974

16. Ishizaka T *et al*: Cell Immunol 113:70, 1974

17. Urban JF *et al*: J Immunol 118:1982, 1977

18. Waldman TA *et al*: N Engl J Med 281:1170, 1969

19. Manouvriey P *et al*: Ann Inst Pasteur/Immunol 136c:187, 1985

20. Suemura *et al*: J Immunol 121:2413, 1978

21. Bazin H *et al*: J Immunol 124:527, 1980

22. Urban JF *et al*: J Immunol 124:527, 1980

23. Bazin H *et al*: J Immunol 121:2083, 1978

24. Katz DH: Lymphocyte differentiation, regulation and recognition. Academic Press, New York, San Francisco, London, pp. 382-470, 1977

25. Okumura K *et al*: J Immunol 106:1019, 1971

26. Taniguchi T *et al*: J Immunol 113:1757, 1974

27. Okudaira H *et al*: J Immunol 111:1420, 1973

28. Hamaoka T *et al*: J Exp Med 138:538, 1973

29. Gershon RK *et al*: Immunology 18:723, 1970

30. Okumura K *et al*: J Immunol 107:1682, 1971

31. Jarrett EE: Immun Rev 41:26, 1978

32. Tada T *et al*: J Immunol 107:579, 1971

33. Taniguchi M *et al*: J Immunol 107:579, 1971

34. Tada T *et al:*J Immunol 108:1535, 1972

35. Hamaoka T *et al:*J Exp Med 138:306, 1973

36. Fox D *et al: I*munol 117:1629, 1976

37. Chiorazzi N *et al*: J Immunol 117:1629

38. Ishizaka K: Ann Rev Immunol 2: 1985

39. Ishizaka K *et al*: Int Arch Allergy appl Immun 77:13, 1985

40. Ishizaka K *et al*: J Immunol 135:1, 1985

41. Paul WE *et al*: Fundamental Immunology, Raven Press, New York, 1984, pp 481-537

42. Mitchison NA: Proc R Soc Lond (Biol)161:275, 1964

43. Weigle WO *et al*: Clin Exp Immunol 9:437, 1971

44. Siskind GW *et al*: J Exp Med 124:417, 1966

45. Mitchison NA *et al*: Immunology 15:531, 1968

46. Coe JE *et al*: J Exp MED LL&:40L, L963

47. Hanson DG *et al*: J Immunol 1234:2337, 1979

48. Battiso *et al*: Proc Soc Exp Biol Med 111:111, 1962

49. Scott DW *et al*: Immun Rev 43:241, 1979

50. Pike BL *et al*: Eur J Immunol 13:214, 1983

51. Nossal GLV *et al*: Proc Natl Acad Sci USA 77:1602, 1980

52 Schrader JW et al: J Exp Med 139:1582, 1974

53. Cooper MD: Contempo Top Immunobiol 1:49, 1973

54. Schrader JW *et al*: J Exp Med 5:808, 1975

55. Abbas AK *et al*: Eur J Immunol 7:667, 1977

56. Boyd AW *et al*: J Exp Med 151:1436, 1980

57. Nossal GTV: Ann Rev Immunol 1:33, 1983

58. Borel Y: Immun Rev 31:1, 1976

59. Pike BL: J Immunol 126:89, 1981

60. Waldschmidt *et al*: J Immunol 131:2204, 1983

61. Lee WY *et al*: Cell Immunol 58:385, 1981

62. Paley RS *et al*: J Immunol 115:1409, 1975

63. Battisto JR *et al*: Nature (London) 212:156, 1966

64. Fidler JM: Immun Rev 50:133, 1980

65. Dorf M *et al*: J Exp Med 149:1336, 1979

66. Naor D: Immun Rev 50:187, 1980

67. Sehon AH: Progr Allergy 32:161, 1982

68. Weinberger JA *et al*: J Exp Med 150:761, 1979

69. Weinberger JA *et al:* J Exp Med 151:1413, 1980

70. Weinberger JA *et al*: J Exp Med 152:161, 1980

71. Sherr DH *et al*: J Exp Med 157:515, 1983

72. Minami M *et al*: J Exp Med 158:1428, 1983

73. Sherr DH *et al*: J Immunol 128:126, 1979

74. Sherr DH *et al*: J Immunol 133:1137, 1984

75. Dorf ME et al: Ann Rev Immunol 2:127, 1984

76. Fidler JM *et al*: J Immunol 111:317, 1973

77. Frey JR *et al*: Clin Exp Immunol 8:131,

78. Frey JR *et al*: Int Arch Allergy appl Immun 42:278, 1972

79. Desamard C: Eur J Immunol 7:646, 1977

80. Desaymard C *et al*: Eur J Immunol 5:541, 1975

81. Coutino A *et al*: Scan J Immunol 3:133, 1974

82. Diener UE *et al*: J Immunol 122:1886, 1979

83. Dinitzis HM *et al*: Proc Natl Acad Sci USA 73:3671, 1976

84. Dintzis RZ *et al*: Proc Natl Acad Sci USA 79:884, 1982

85. Dintzis RZ *et al*: J Immunol 131:2196, 1983 63:1, 1980

86. Dintzis RZ *et al*: J Immunol 135:423, 1985

87. Aldo-Benson M *et al*: J Immunol 112:1793, 1974, 1977

88. Aldo-Benson M *et al*: J Immunol 116:223, 1976 1981

89. Pan D *et al*: Fed Proc 35:433 (Abstract), 1976

90. Hubbaard DA *et al*: J Immunol 126:407, 1981

91. Lee WY *et al*: J Immunol 126:407, 1981

92. Lee WY *et al*: Int Arch Allergy appl Immun 63:1, 1980

93. Lee WY *et al*: J Immunol Lett 2:347, 1981

94. Holford-Strevens V *et al:* Int Arch Allergy appl Immunol 67:109, 1982

95. Zeiss CR *et al*: J Allergy Clin Immunol 60:96, 1977

96. Sale SR *et al*: J Allergy Clin Immunol 68:188,1981

97. Lee WY *et al*: Eur J Immunol 11:13, 1981

98. Hebert FA *et al*: Chest 76:546, 1979

99. Patterson R *et al*: Am Rev Resp Dis 120:1259, 1979

100. Turner ES *et al*: Clin Exp Immunol 39:470, 1980

101. Zeiss CR *et al*: Int Arch Allergy appl Immun 61:380, 1980

102. Patterson R *et al*: Int Arch Allergy appl Immun 39:459, 1970

103. Akiyama K *et al*: J Immunol 133:3286, 1984

104. Ceska M *et al*: J Allergy Clin Immunol 49:1, 1972

105. Kelly KA *et al*: J Immunol Meth 39:317, 1980

106. Mishell BB & Shiigi SM: Selected methods in cellular immunology. San Franciso, WH Freeman & Company, 1980

107. Shaw's Farm, Blackhorn, Bicester, Oxon: Information sheet (anti-Thy 1.2 F7D5 monoclonal IgM antibody)

108. Cunningham AJ *et al*: Immunol 14:599, 1968

109. Salvaggio JE: J Allergy clin Immunol 70:5, 1982

110. Davies RJ *et al*: J Allergy clin Immunol 60:93, 1977

111. Butcher BT *et al*: J Allergy clin Immunol 64:146, 1979

112. Butcher BT *et al*: J Allergy Clin Immunol 58:89, 1976

113. Tse KS *et al*: Clin Allergy 12:249, 1982

114. Karol MH *et al*: J Occup Med 21:354, 1979

115. Malkiel S *et al*: J Allergy 23:352, 1952

116. Klinman *et al*: J Immunol 126:1970, 1981

117. Tew JG *et al*: Immun Rev 53:175, 1980

118. Zheng SS *et al*: unpublished data.

119. Schwenk RJ *et al*: J Immunol 123:2791, 1979

120. Watanabe MR *et al*: Cell Immunol 79:345, 1983

121. Humphrey *et al*: Eur J Immunol 11:221, 1981

122. Weissman I: J Exp Med 126:291, 1967

123. Liu FT *et al*: J Allergy clin Immunol 66:322, 1980

124. Lee WY *et al*: J Immunol 116:1711, 1976

125. Parks DE & Weigle WO: Immun Rev 43:217, 1979

126. Tzartos SJ *et al*: Proc Natl Acad Sci USA 77:755, 1980

127. Lennon VA *et al*: Proc Natl Acad Sci USA 82:8805, 1985