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SUPPRESSION OF THE IgE ANTIBODY RESPONSE WITH MODIFIED AND SOLUBLE ANTIGEN

by

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

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TO THE LORD GOD

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ABSTRACT

With an aim to devise immunologically specific methods for the suppression of reaginic antibody responses, two systems have been successfully developed by the use of (i) isologous murine gamma globulins (MγG) as nonimmunogenic carriers for the synthesis of tolerogenic derivatives of protein antigen (ovalbumin, OA), (ii) soluble protein antigen (dog albumin, DA). Indeed, the results of this investigation demonstrated that treatment with conjugates of OA-MγG, of normal mice or of mice presensitized with DNP-OA to a state of immediate hypersensitivity, suppressed the ability of these mice to produce anti-OA IgE antibodies, and of IgE antibodies to the hapten, 2,4-dinitrophenyl (DNP), when the latter was presented to the immunological system with OA as a carrier. However, presentation of this hapten on a different carrier led to normal anti-DNP and anti-carrier IgE responses, suggesting that this type of tolerance was highly carrier-specific. Moreover, experimental results in this study indicated that this mode of immunosuppression was at least in part mediated by suppressor cells. It was shown also that transfer of spleen cells from tolerant mice into normal and primed syngeneic recipients resulted in the suppression of primary and secondary IgE responses of the respective recipients on immunization with a sensitizing dose of DNP-OA.

In a parallel study, soluble DA induced a specific, long-lasting unresponsive state in normal mice or in mice presensitized with DA in spite of 2 subsequent sensitizations with DA. This state was mediated, at least partially, by the dampening of T helper cell function since mice primed with DNP-OA were unable to form anti-DNP IgE antibodies after their treatment with soluble DA. Moreover, in an adoptive transfer system, T cells from treated animals were not capable of providing the necessary helper function to B cells from mice primed with DA in order to elicit

anti-DA, IgE response. In contrast, T cells from mice primed with DA provided helper function to B cells either from treated mice or mice primed with DA. It was also shown that antigen specific Ts cells were at least partially responsible for the unresponsive state. In addition, a factor extracted from the Ts cell, abrogated the anti-DA IgE response in an adoptive transfer system.

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ABBREVIATIONS

$Al(OH)_3$: aluminum hydroxide

ALS : anti-mouse lymphocyte serum

anti- : antibody

α-A : α-amylase

Asc : *Ascaris suum* extract

B : bursa of Fabricius equivalent cell

B_ε : bursa of Fabricius equivalent cell producing IgE

BBS : borate buffered saline

BGG : bovine gamma globulin

BPO : benzylpenicilloyl group

BSA : bovine serum albumin

$B_6D_2F_1$: $(C_{57}Bl/6 \times DB\ A/2)F_1$ mice

CON A : concanavalin A

C' : complement

CPM : counts per minute

CY : cyclophosphamide

DA : dog albumin

DNP : dinitrophenyl group

D-GL : copolymer of D-glutamic acid -D- lysine

FCA : Freund's complete adjuvant

FCS : fetal calf serum

FIA : Freund's incomplete adjuvant

GAT : copolymer of L-glutamic acid, L-alanine, L-tyrosine

γ : gamma

H.A. : hemagglutination

H-2 : major histocompatibility complex of the mouse

HSA : human serum albumin

Ig : immunoglobulin

IgG : immunoglobulin of the G class

IgE : immunoglobulin of the E class

^{125}I : radioactive istotope of iodine, 125

i.p. : intraperitoneally

Irgene : immune response gene

I-Jgene: gene located in the J region of the H-2 complex

i.v. : intraveneously

KLH : keyhole limpet hemocyanin

MEM : minimal essential medium

2ME : 2- mercaptoethanol

ml : milliliter

mg : milligram

MγG : mouse gamma globulin

MIgG : mouse immunoglobulin of the G class

MIgM : mouse immunoglobulin of the M class

MSA : mouse serum albumin

NMS : normal mouse serum

OA : ovalbumin

OA-MγG : ovalbumin conjugated to MγG

OA-RγG : ovalbumin conjugated to RγG

PCA : passive cutaneous anaphylaxis

PEG : polyethylene glycol

PFC : plaque forming cells

PBS : phosphate buffered saline

PVA : polyvinyl alcohol

RAG : water soluble extract of ragweed pollen

RYG : rabbit gamma globulin

RNase : ribonuclease

RPM : revolutions per minute

SRBC : sheep red blood cells

T : thymus derived cells

θ : theta antigen

Tbc : mycobacterium

Tca : trichloroacetic acid

Th : thymus derived helper cells

Ts : thymus derived suppressor cells

UD-C : urea denatured carrier

µg : microgram

µl : microliter

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

It has been well established that common forms of hypersensitivity of the immediate type are mediated primarily by IgE antibodies, produced by allergic individuals in response to a wide spectrum of allergens. In an attempt to devise effective treatments based on the current knowledge of the cellular interactions regulating the formation of antibodies in general (1) and of IgE antibodies in particular (2-5), many studies have been directed in recent years with a view to developing specific methods for the immunosuppression of IgE antibodies. Thus, the administration of conjugates, consisting of haptens covalently linked to nonimmunogenic molecules, specifically abrogated the IgE response to the respective antigen (reviewed in 6). In other approaches, protein antigens denatured with urea (5) or chemically modified by acetoacetylation (2) suppressed also specifically the IgE responses. Moreover, pollen allergens were rendered immunosuppressive by treating the allergens with either formaldehyde (7) or glutaraldehyde (8,9). The induction of immunological unresponsiveness in the IgE class of antibody employing the various protocols described above, resulted, in some instances, in the generation of thymus-derived suppressor cells (Ts). Tolerance due to receptor blockade or to clonal abortion of antibody producing cells (B) was postulated in some of the experimental systems.

The use of antigens linked to the nonimmunogenic carrier, the copolymer of D-glutamic acid, D-lysine (D-GL) has been recently reported by Liu et al (10,11) in which they demonstrated that the conjugates preferentially affected thymus derived helper cells (Th) without inducing

detectable Ts cell activity. However, the effect of protein antigens linked to isologous gamma globulins on the production of IgE antibodies in animals has not been reported. Previously, Lee and Sehon (reviewed in 6) clearly demonstrated that haptens linked to isologous gamma globulins suppressed the primary and secondary anti-hapten IgE responses.

The attempts in dampening the reaginic antibody response by the administration of soluble antigen has met with little success. Various investigators (6,11,12) demonstrated that the IgE response was only transiently suppressed by soluble antigen. In this laboratory preliminary results indicated that soluble dog albumin (DA) as well as conjugates of DA coupled to polyethylene glycol (DA-PEG) suppressed the anti-DA IgE response. Therefore, two systems were developed in the present study, in order to determine the effect of ovalbumin-murine isologous gamma globulin (OA-MγG) conjugates and soluble DA on the reaginic response.

Indeed, the results of this investigation demonstrated that treatment with conjugates of OA-MγG of normal mice or of mice presensitized with DNP-OA to a state of immediate hypersensitivity, suppressed the ability of these mice to produce anti-OA IgE antibodies and IgE antibodies to the hapten, 2,4 -dinitrophenyl (DNP), when the latter was presented to the immunological system with OA as a carrier. However, presentation of this hapten on a different carrier led to normal anti-DNP and anti-carrier IgE responses, suggesting that this type of tolerance was carrier-specific. Moreover, experimental results in this study indicated that this mode of immunosuppression was at least in part mediated by suppressor cells.

In the parallel study, soluble DA was found to induce a specific, long-lasting unresponsive state in normal mice or mice presensitized with

DA even upon two subsequent sensitizations with DA. This state was at least partially mediated by the dampening of Th cell function since mice primed with DNP-DA, after their treatment with soluble DA were unable to form anti-DNP IgE antibodies. Moreover, in an adoptive transfer system, T cells from animals treated with soluble DA were not capable of providing the necessary helper function to B cells from mice primed with DA in order to elicit anti-DA IgE response. In contrast, T cells from mice primed with DA provided helper function to B cells either from treated mice or mice primed with DA. It was also shown that antigen specific Ts cells were at least partially responsible for the unresponsive state. In addition, a factor extracted from the Ts cell abrogated the anti-DA IgE response in an adoptive transfer system.

LITERATURE REVIEW

The humoral responses to antigens are mainly categorized into two, depending upon the type of antigen used for elicitation of the response, namely thymus dependent or independent. Antigens which depended on thymus derived (T) cell interaction with bursa of Fabricius-derived cells (B) for antibody synthesis were categorized as T-dependent whereas, T-independent antigens induced an immune response without the apparent participation of T lymphocytes. The early studies of Humphrey et al (13) revealed that some antigens elicited immune responses to the capsular polysaccharide of type III pneumoccoci in the absence of T cells. However, Claman and co-workers were among the first to provide evidence for T-B cell interactions in the humoral responses of mice to the T-dependent antigen, sheep red blood cells (SRBC) (14-16). Thus, lethally irradiated recipients were injected intraveneously with varying numbers of either spleen, thymus, bone marrow cells or a mixture of thymus and bone marrow cells, from normal or immune syngeneic donors, followed by antigenic challenge with SRBC. The recipients were bled at various times after cell transfer, for the determination of hemolytic activity in their sera. Hence, Claman and associates postulated from their results that the marrow population contained "effector cells" capable of producing antibody, but only in the presence of "auxiliary cells" present in the thymus population.

Support for this interpretation was forthcoming from Miller and Mitchell's laboratory (17). They reported that bone marrow cells of neonatally thymectomized mice were as effective as bone marrow cells of normal mice in restoring immunological responsiveness of heavily irradiated mice with intact thymuses. Furthermore, the immune response to SRBC was restored upon the administration of allogeneic thoracic duct or thymus

lymphocytes to neonatally thymectomized recipients. However, anti-H2 sera against host cell antigens, inhibited in vitro plaque forming cell (PFC) response to SRBC but anti-H2 antibodies directed against donor thoracic duct or thymus cells did not affect the response (18). In addition, they also established that precursors were indeed derived from the bone marrow population and moreover, that thymus cells recognized and reacted specifically with antigen, but performed an auxiliary role in the production of antibody by B cell precursors.

The pioneering work of Mishell and Dutton (19,20) and Marbrook (21) in which they elicited immune responses in vitro provided a useful tool in the study of specific cellular interactions to particulate antigens i.e. SRBC, and to soluble antigens, i.e. human serum albumin (HSA). Hence, the role of adherent and non-adherent cells in immune responses was studied (22,23). Subpopulations of splenic cells were obtained on the basis of their relative abilities to adhere to plastic or glass surfaces. These populations of cells developed a poor primary in vitro anti-SRBC response; however, remixing the two populations, the response was restored. In addition, the cell type in the adherent and nonadherent populations required for the in vitro response was identified as the macrophage and B,T lymphocytes respective. Furthermore, spleen cells from adult thymectomized, lethally irradiated mice reconstitued with syngeneic bone marrow cells were not capable of producing an in vitro response to SRBC. However, the in vitro response was restored upon reconstituting the mice with a thymus graft.

These initial studies which demonstrated the requirement for cellular interactions in the response to SRBC were followed by analogous findings in the development of humoral in vitro responses to soluble

protein antigens and perhaps more importantly to hapten-carrier conjugates (24-26). Landsteiner (27) introduced defined haptenic determinants onto immunogenic carriers and provided a valuable tool for the analysis of specific interactions between antigens and specific cells of the immune response. Briefly, immunization with a hapten-carrier conjugate elicited an anti-hapten response only when the hapten was coupled to an immunogenic carrier; nonimmunogenic carrier served only poorly or not at all, (28,30). Moreover, the induction of optimal hapten-specific secondary responses required the second challenge with the same hapten-carrier conjugate as that used for the primary immunization (31). The anti-hapten antibodies produced by such immunizations were highly specific for the haptenic determinant employed. Therefore, the assumption was made that the specificity of the antibody accurately expressed the specificity of the antigen-binding receptor molecules on the antibody forming cell precursor. Thus, the latter observations suggested the operation of an additional recognition mechanism for the carrier molecule. Indeed, cooperative interactions which were essential for the development of anti-hapten immune responses, were demonstrated between distinct lymphocytes, specific for carrier and haptenic determinants.

The first direct evidence for cooperative participation of two cells with distinct determinant specificities in the humoral response to hapten-carrier conjugates was initially obtained by Mitchison (32). Briefly, spleen cells from syngeneic donors, previously immunized either with a hapten-carrier conjugate or a second unrelated carrier injected together into irradiated recipients, produced an anti-hapten response when sensitized with the hapten heterologous carrier conjugate. In contrast, recipients of either cell population, which were immunized

with the heterologous hapten carrier conjugate, did not produce any anti-hapten response. Therefore, the cooperation of carrier-specific cells and anti-hapten antibody producing cells was essential for maximal anti-hapten antibody production.

Raff (33) demonstrated that the carrier-specific cooperating cells or "helper cells" were induced in the thymus, whereas anti-hapten antibody forming cells were not. Thus, Raff employed the identical protocol as described by Mitchison (32) and demonstrated that the carrier specific helper cells were T cells since the cells were sensitive to anti-theta and complement whereas the anti-hapten antibody producing cells were not sensitive to the treatment. Hence, by extension, B lymphocytes were considered to be the hapten antibody producing cells.

Conclusive evidence which showed that the B lymphocytes lineage were the progenitors of antibody secreting cells, came from studies using anti-sera specific for the mouse bone marrow lymphocyte marker (MBLA) in the mouse. Niederhuber et al (34) provided evidence that MBLA determinants existed on precursors of antibody forming cells, by employing an adoptive transfer studies of cooperative immune response to hapten-carrier conjugates. It was shown that hapten-specific B cells were susceptible to the cytotoxic effects of anti-MBLA antibodies but not anti-theta antibodies.

The same cooperation phenomenon between carrier-specific T cells and hapten-specific B cells was shown by a somewhat different approach and involving supplemental immunization of the intact animal with free carrier. Thus, rabbits immunized with p-azobenzene sulfonic acid (sulfanil) derivative of bovine serum albumin (BSA) made significant secondary anti-sulfanil antibody responses to sulfanil-human gamma globulin (HGG), when the rabbits had received a supplemental intervening

immunization with the free, unconjugated carrier, HGG (35). These results were subsequently confirmed by Katz (36,37) in rabbits and in guinea pigs.

A subset of T cells were involved also in the negative regulation of the immune response. These cells were first described by Gershon (38-40) and have been named Ts cells. Two broad categories of Ts cells are defined on the basis of functional effects of the cells, i.e. specific and nonspecific cells. The antigen specific Ts cells were defined as such, since, their induction and biological effects were restricted to response related to specific antigenic determinants. On the other hand, nonspecific Ts cells are not restricted in their effects or function, irrespective of whether or not the Ts cells were specifically activated by an antigen (41).

Gershon and Kondo (42,43) observed in mice the activity of Ts cells in the development of immunological unresponsiveness to SRBC. The effects of Ts cells tended to be essentially the opposite of Th cell. Among the first studies which demonstrated a dualistic influence of carrier-specific T cells in response to hapten-carrier conjugates were those performed by Katz et al (44) and Tada (45). Thus, guinea pigs were primed with soluble DNP-OA on day 0 and one week later, the various groups were immunized with BGG or keyhole limpet hemocyanin (KLH) or a mixture of BGG and KLH emulsified in Freund's complete adjuvant (FCA) (44). Three weeks later, the guinea pigs were injected intraperitoneally (i.p.) with 10 mg of soluble BGG or KLH followed one day later by a secondary challenge with DNP-BGG. The supplemental immunization with BGG in FCA induced a population of BGG-specific T cells capable of regulating the response to DNP-BGG. The net effect resulted in a very strong secondary

anti-DNP response. However, administration of soluble BGG 1 day prior to the secondary immunization abrogated the anti-DNP response of the recipients. The unresponsive state was antigen specific since administration of unconjugated KLH to guinea pigs preimmunized with BGG in FCA failed to suppress the response. However, guinea pigs treated with KLH in FCA and resensitized with DNP-BGG showed clearly a depressed anti-DNP response demonstrating a nonspecific dampening of the anti-DNP response by KLH.

Moreover, a specific unresponsive state was induced in rats administered the carrier prior to sensitization with the homologous hapten-carrier conjugate, whereas rats challenged with a heterologous hapten-carrier conjugate produced a primary anti-hapten antibody response (45). The unresponsive state induced in these animals was mediated by antigen specific Ts cells. Hence, rats treated with KLH and challenged with DNP-BGG produced an anti-DNP antibody response.

Moreover, Tada and associates extracted a soluble suppressive factor from the Ts cell. The factor did not contain any immunoglobulin determinants and had a molecular weight between 35,000 and 60,000 daltons (46,47). These early results prompted Tada and his collaborators to study the factor more closely. They opted for an in vivo cell transfer system employing inbred mice and extracted a soluble factor from Ts cell induced in mice primed earlier with KLH (47-49). This Ts cell factor had very similar effects and properties as the Ts cell factor obtained in rats.

The genetic nature of the Ts cell factor was studied by using various inbred strains of mice differing in H-2 haplotypes and the genetic requirements for the interaction of suppressor and acceptor molecules were elucidated. Tada and co-workers first demonstrated by employing allo-

antisera to the various subregions, that the unique Ts cell factor was a product of a restricted subregion which was later defined as I-J subregion of the I region of the H-2 complex (50,51).

Moreover, they clearly demonstrated that: 1) the Ts cell factor was effectively absorbed with alloantisera directed to I region of the H-2 complex; 2) alloantisera against histocompatibility antigens coded for by K or D region genes did not absorb the Ts cell factor; 3) the absorbing capacity of alloantisera did not correlate with the anti-Ia specificities which had been determined by cytotoxic activity for B cells; 4) antisera containing specificity for I-J subregion gene products absorbed the suppressive activity, whereas those lacking I-J specificity did not; 5) the Ts cell factor obtained from one strain of mice effectively suppressed the responses of other strains sharing the same I-J subregion and 6) the acceptor site for the Ts cell factor was also postulated to be coded for by genes in the I-J subregion. It was suggested that the I-J subregion genes coded for complementary cell surface molecules on different subsets of lymphoid cells through which suppressive interaction was effectively achieved (52,53).

One of the more striking systems involved in the activity of Ts cells has been that of the genetically controlled response to the synthetic terpolymer, GAT. Antibody responses to GAT are controlled by an autosomal dominant Ir gene mapping in the I region of the H-2 complex. Gershon et al (54) initially postulated that the cellular basis for unresponsiveness in nonresponder strains of mice to GAT was related to the existence of Ts cells inhibiting the capacity of such animals to produce antibodies to GAT. The hypothesis was based upon the ability of GAT to stimulate DNA synthesis in vitro, following primary immunization

of nonresponder mice with GAT. No secondary stimulation of DNA synthesis occurred in vitro with spleen cells from the nonresponder mice, however responder mice exhibited a marked secondary response.

Subsequently, Kapp and colleagues (55-61) demonstrated specific Ts cells for GAT which regulated the anti-GAT antibody response. Responder strains of mice developed GAT-specific IgG and IgE responses in vivo and IgG responses in vitro, following stimulation with GAT alone, whereas, nonresponder mice failed to develop GAT-specific responses with GAT unless these animals were immunized with GAT complexed to methylated bovine serum albumin (GAT-MBSA). However, the injection of GAT into these mice in appropriate doses, specifically decreased the ability of the recipients to develop a GAT-specific response to subsequent challenge with the normally immunogenic form of the antigen, GAT-MBSA; responder mice were not affected in this manner (58). The unresponsive state induced in nonresponder mice, was the result of activation of GAT-specific Ts cells and not due to the development of B cell tolerance.

Furthermore, Kapp et al (62) found that although nonresponder mice did not reveal GAT-specific Th function, the mice were capable of showing limited GAT-specific Th activity under certain circumstances. Thus, nonresponder mice injected with GAT bound to syngeneic macrophages, developed radioresistant Th cells and the treatment of these mice with either low doses of cyclosphosphamide or antiserum to the I-J subregion determinants, which eliminated Ts activity induced a small but significant, PFC response to GAT indicating weak Th cell function. Therefore, nonresponders were incapable of producing anti-GAT antibody, since Ts cells were preferentially activated by the injection of GAT. Moreover, the macrophages were implicated as mediating the selection of Ts versus Th cells. The

depletion of macrophages from responder spleen cells followed by an immunogenic challenge of GAT in an in vitro culture system, led to the development of highly active antigen specific Ts cells in the nonadherent population (63). The induction of Ts cells was dose dependent since increasing the concentration of GAT to 5-10 times the normal immunogenic concentration, induced GAT-specific Ts cells in responder spleen cells containing macrophages. In addition, nonresponder spleen cells developed GAT-specific Ts cells at much lower concentrations than the nonadherent responder cells. Therefore, the triggering of the Th cells in preference to Ts cells depended upon antigen presentation by the macrophage.

The discussion in the previous section, was focused on some of the general characteristics of the humoral responses. The IgE antibody response will be discussed in great detail in the succeeding pages. The system has particular importance because of its clearly defined role in the pathogenesis of human allergic diseases. Furthermore, a clear understanding of the regulatory mechanism controlling the reaginic response will provide valuable clues and insights as to the nature of the general regulatory events governing not only the IgE response but also the immune system at the genetic, cellular and molecular levels.

IgE antibodies are present only in minute quantities in the serum as compared to other classes of immunoglobulins and has a very short life in serum (64-66). Therefore, IgE antibodies are continuously synthesized in the respiratory and gastrointestinal mucosa as well as in regional lymph nodes under normal circumstances (64-66). This observation suggested an important role for these antibodies in protecting the host against foreign agents i.e. pollens and parasites, likely to invade these sites. So far the predominant function of IgE antibody is to fix to tissue mast cells or basophils resulting, upon exposure to

multivalent antigen, in the release of vasoactive substances such as histamine, slow-reacting substance of anaphylaxis, the eosinophil chemotactic factor of anaphalaxis and bradykinin (67).

The nature of antigens usually capable of readily stimulating IgE production are distinctive from other conventional antigens capable of stimulating responses of other immunoglobulin classes. Grass pollens, certain fungi, allergens of food, parasitic nematodes and trematodes usually induce high and persistent levels of IgE antibodies, whereas most serum proteins, bacteria and viruses, despite the fact that they are highly immunogenic for IgM and IgG antibody responses, do not usually induce a reaginic response. Simple haptens such as dinitrophenyl (DNP) and benzylpenicilloyl (BPO) groups induced high IgE titers when conjugated to appropriate carriers, whereas hapten conjugates of T-independent antigens usually do not stimulate IgE antibody responses. For the induction of IgE antibodies in experimental animals, particular attention must be given to the dose and adjuvant. The general rule appears to be that high doses of antigen and certain adjuvants used for inducting IgG and IgM antibodies such as FCA are particularly unfavorable to generate IgE antibodies.

The synthesis of IgE antibody depends upon the animal species, the nature of antigen and the adjuvant employed. Hence, Mota (68) and Binaghi and Benacerraf (69) induced primary antibody responses in rats injected with protein antigens together with Bordetella pertussis. However, the responses were transient in nature and the animals failed to develop secondary IgE antibody responses upon subsequent challenge, although secondary IgG antibody responses to the antigen were elicited. On the contrary, rats infected with the nematode Nippostrongylus brasiliensis, produced an IgE antibody response which persisted at high

levels for many months eventhough the worms were completely expelled from the host (70). In addition, a secondary IgE antibody response was elicited in these animals by reinfecting the host with the same parasite (71). In contrast, the deliberate immunization of rats with extracts of the parasite induced a primary but transient IgE antibody response and resensitizing the animals with the extract failed to elicit a secondary IgE response.

Levine and Vaz (72) and Vaz et al (73), reported that persistent and high titers of IgE antibodies were produced in mice with extremely low doses of antigen (1 µg or less) precipitated on aluminum hydroxide gel. However, high doses of the same antigen elicited little or no IgE antibody production, although high levels of IgG antibody were induced with the high doses of antigen (72). Moreover, the primary IgE response induced in mice with low doses of antigen was boosterable and secondary response were elicited upon challenge.

The capacity of mice to develop high titers of persistent IgE antibody and secondary responses was genetically controlled and linked to the H-2 complex. This was first suggested by studies of Revoltella and Ovary (74) and clearly demonstrated by Levine and Vaz (72). Subsequently Dorf et al (75) and Dessein et al (76) defined the existence of an H-2 linked immune response gene (Ir), controlling antibody production to ragweed pollen extract (RAG) and to GAT. Moreover, the Ir gene(s) controlling the antibody production were mapped to the I region of H-2 complex by studying the responses of the appropriate H-2 recombinant inbred mice. In man, an analogous Ir gene controlling responses to RAG and linked to the major histocompatibility complex (HLA) has been postulated by Levine et al (77) and by Marsh et al (78).

Several studies on IgE antibody synthesis indicated the participation of T lymphocytes in such responses. Various investigators were able to induce anti-hapten antibody responses of the IgE class in several animal species following immunization with hapten-protein conjugates (68,69,79,80). The role of T lymphocytes in IgE antibody synthesis was investigated in the following manner. Animals were preimmunized with unconjugated carrier which produced an augmented primary IgE antibody response to a DNP conjugate when reimmunized with the homologous carrier coupled with the hapten. Thus, carrier determinants were first recognized by carrier specific cells which interacted with hapten specific B cells to induce the formation of anti-hapten antibodies (81-83). Moreover, a specific unresponsive state was induced also in adult rats rendered tolerant as neonates to the extract of Ascaris suum (ASC). However, this state was reversed upon the injection of thymocytes from normal or carrier-preimmunized donors, thus enabling the recipients to produce IgE antibodies in response to stimulation with DNP-Asc.

Similar observation in the regulation of the IgE antibody response was reported also by Okudaira and Ishizaka (85) and Okumura and Tada (86). For example, mice primed with DNP-Asc, which ordinarily failed to develop secondary anti-DNP IgE responses to a challenge with a DNP-OA produced a typical secondary anti-DNP antibody response if a supplemental immunization with OA was given. The requirement for T lymphocytes in the development of IgE antibody response was demonstrated in neonatally thymectomized rats which were unable to produce IgE antibody response upon subsequent immunization with a hapten-carrier conjugate (86,87). However, the response was reconstituted by supplementing the neonatally thymectomized rats with normal or carrier primed thymocytes

(89). Such results indicated cooperation between carrier specific cells and hapten specific memory cells was needed for the induction of anti-hapten responses.

The development of adoptive cell transfer systems has led also to the demonstration of T and B cell cooperation in IgE antibody responses in the mouse. Hamaoka et al (88) with the aid of an adoptive cell transfer system, induced IgE antibodies to DNP in irradiated recipients of spleen cells from syngeneic donors sensitized in an appropriate manner to produce IgE antibodies. Similarly, Hamaoka (82) demonstrated cooperative interactions between carrier specific and hapten specific mouse spleen cell populations in the production of IgE antibodies. Moreover, they established that theta-bearing T lymphocytes functioned as the carrier-specific helper cells for IgE antibody production. Michael and Bernstein (89) corroborated these findings in studies performed in congenitally athymic nude mice. These mice were unable to produce IgE antibodies upon stimulation with OA, but the defect was corrected by the administration of thymocytes or the grafting of a thymus.

The capacity to suppress antibody response of the IgE class is of practical importance in terms of effective therapeutic maneuvers in clinical allergy. In classical studies performed by Tada and his colleagues (90,91) rats were immunized with antigen and adjuvant to develop IgE responses which were moderate in magnitude, transient in nature and not possible to boost by a second sensitizing dose of antigen. However, exposure of these rats to low doses of irradiation either shortly before or after immunization with antigen and adjuvant greatly enhanced the production of IgE antibody while at the same time suppressing responses of other antibody classes. The IgE responses

were not enhanced in magnitude but persisted for a considerably longer period of time as compared to the IgE response in normal nonirradiated animals. Identical results in terms of IgE antibody responses were obtained following adult thymectomy and/or splenectomy of rats prior to immunization with hapten-carrier conjugate (86). However, no diminution of IgM or IgG responses was detected in these animals indicating that the augmentation was not due to defective feedback regulation by serum IgE on the production of IgE antibodies. The results suggested that these maneuvers failed to inhibit the induction of IgE antibodies and eliminated the dampening mechanism of the IgE response, which resulted in the enhancement and prolongation of IgE antibody synthesis.

These investigators also studied the effects of a variety of immunosuppressive drugs on IgE antibody responses in rats (92). The administration of the inhibitor of DNA synthesis, 5-bromouridine deoxyriboside or actinomycin D, an inhibitor of DNA dependent RNA synthesis resulted in a marked enhanced and prolonged IgE response. In addition, the alkylating agent, cyclophosphamide, delayed significantly the IgE response but a striking high IgE antibody titer was detected in animals pretreated with the drug before immunization. However, the injection of cyclophosphamide after immunization led to a complete suppression of the humoral response including the IgE class. The treatment of rats with cortisone either prior to or after immunization, led either to a persistent moderate production or to a slight inhibitory effect of IgE antibody responses. Furthermore, treatment of rabbtis with 2-mercaptopurine enhanced the IgE antibody response to antigen (93).

Ts cells were shown also to be involved in the regulation of IgE antibody responses (94). The enhancement of IgE antibody response

observed in irradiated rats was terminated by passive transfer of either thymocytes or spleen cells from syngeneic, carrier-primed rats. Thus, a population of carrier primed lymphocytes abrogated the IgE response in these rats. The suppressive effects of carrier-specific T cells in animals hyperimmunized with either hapten or carrier in FCA and subsequently reimmunized with hapten-carrier together with Bordetella pertussis were shown by Tada et al (84). The results indicated that preimmunization with carrier alone in FCA led to the production of carrier-specific Ts cells capable of inhibiting the IgE antibody response to hapten or carrier. Similar observations were made by Hamaoka (88) in which the enhancing effects of low dose irradiation on IgE production in mice was examined. The magnitude of secondary IgE antibody responses obtained in irradiated recipients of primed spleen cells was substantially higher than the responses elicited in the donors even after repeated immunization with optimal doses of antigen. The high response of the irradiated recipients was thought to be due either to a non-specific stimulus existing in the environment or irradiation destruction of tissue mast cells to which IgE molecules fixed to. The investigators have since changed the interpretation of their data and believed that the results reflected a similar phenomenom observed in the rat by Tada and associates (91,92).

Further evidence in support of the involvement of carrier-specific Ts cells was provided by Kind and coworkers (95,96) who demonstrated that the injection of equine anti-mouse lymphocyte serum (ALS) without adjuvant into mice initiated IgE antibody responses against equine serum proteins. Furthermore, Kind and Macedo-Sobrinho (97) employed a combination of ALS plus irradiation and obtained a persistent IgE antibody response against ALS determinants. However, the combination

of thymectomy plus ALS treatment was ineffective in inducing a long-lasting IgE antibody response.

The effect of irradiation on primary and secondary IgE responses in the mouse to hapten-carrier conjugates was studied by Fox et al (98) and Chiorazzi et al (99). The results demonstrated a differential sensitivity of IgE and IgG B lymphocytes to the effects of ionizing irradiation, in that the former cell tended to be considerably more resistant to detrimental effects of such treatments than do cells of the IgG class. It was noted that in these mice an enhanced IgE antibody response was elicited by the irradiation treatment whereas, no comparable IgE enhancement was made in the mice. Similar results were obtained by the administration of cyclophosphamide. Unprimed or primed mice developed 4 to 16 fold higher levels of antigen specific IgE antibodies than controls when exposed to doses of irradiation, from 50 to 200 rads, immediately prior to antigenic stimulation. Similarly, primed mice pretreated with cyclophosphamide 2 days prior to challenge produced high levels of specific IgE antibodies.

The cellular site of action of irradiation and cyclophosphamide was determined with the use of an adoptive transfer system utilizing carrier-primed recipients as hosts for spleen cells of syngeneic mice which had been primed with a hapten conjugated with a heterologous carrier (99). Such experiments demonstrated conclusively that ionizing irradiation and cyclophosphamide, exerted their effects by affecting the carrier primed host T cell population and not by affecting the components of the transferred B cell population. Moreover, the suppressive activity was non-specific since unrelated responses were similarly dampened.

Watanabe et al (100,101) demonstrated nonspecific suppressor cells in normal low IgE antibody responder mice and the suppressive function was inherited by a single Mendelian gene not linked to the H-2 complex. These nonspecific suppressor cells suppressed ongoing IgE antibody responses and were shown to be T cells in terms of their susceptibility to treatment with anti-theta serum and complement and by their capacity to transfer the suppression. The Ts cells in SJL mice, were phenotypically characterized as an $Ly\text{-}1^{+}2^{-}$ which is different from the phenotype of the antigen-specific suppressor cells which is Lyl^{-}, $Ly\text{-}2^{+}3^{+}$ (102,103).

Recently, Tamura and Ishizaka (104) and Schwenk et al (105), characterized Ts cells which regulated both persistent and transient IgE responses. The injection of different doses of antigen led to a variety of Th, Ts and B cell activities. A minimum dose (0.05 µg) of immunogen injected into mice was favorable for the induction of Th cell activity and B cell memory whereas, 1 or 10 µg of antigen led to favorable development of B cell memory. The low response to high doses of antigens was attributed to antigen specific Ts cells (104). Nevertheless, it was not possible for the authors to rule out the possibility that IgG antibodies were suppressing the IgE response (105). However, the authors provided evidence with the use of an adoptive transfer system that the unresponsive state was at least partially mediated by Ts cells.

Gollapudi and Kind (106) induced high levels of IgE antibodies in low responder SJL mice by injecting mixture of antigen and Concanavalin A (Con A). The results indicated an in vivo activation by Con A of T cells to produce soluble factors, which in turn stimulated IgE B cells which had bound antigen.

Nonspecific suppression of IgE responses was also reported by Kishimoto et al (107). The injection of hapten-conjugated mycobacterium (H-Tbc) in FCA 2 weeks prior to immunization with the hapten conjugated to an unrelated protein carrier incorporated in aluminum hydroxide gel resulted in the complete suppression of the IgE anti-hapten response, whereas the IgG response was enhanced. Moreover, the anti-carrier response was also significantly depressed. However, the anti-carrier IgE response of mice treated with H-Tbc but immunized with heterologous hapten conjugated to the homologous carrier was not suppressed.

Suemura et al (109) demonstrated selective suppression of anti-hapten IgE response by H-Tbc primed T cells by using an in vitro culture system of murine spleen cells. Depletion of T cells by anti-theta treatment removed the suppressive activity of H-Tbc primed cells, whereas, depletion of B cells by anti-Ig columns did not. The unresponsive state induced in vitro by these cells was dose dependent and radiosensitive. Cell free supernatants (CFS) from the culture of hapten-Tbc primed cells with either hapten-carrier pulsed macrophages or hapten-carrier coupled Sepharose beads showed suppressor activity of anti-hapten IgE responses but did not affect IgG anti-hapten responses. The suppressive activity of CFS was not absorbed by hapten-carrier or anti-Ig coupled Sepharose immunosorbents, indicating that the IgE class specific suppressor factor was not antigen specific but antigen was needed for the triggering of cells and did not possess Ig determinants. The IgE class specific factor was absorbed with allo-antiserum directed to the subregion between I-A and I-E of the H-2 complex and did not exert its function across an H-2 barrier. The suppressor activity of the factor was removed by absorption with B cells primed with hapten but not with T cells. The

factor inactivated also hapten-primed B cells i.e. the B cells were not able to cooperate with carrier primed T cells in an adoptive cell transfer system. Therefore, it was concluded that the target cell for the suppressor factor was B cells (109,110).

Katz and his colleagues (111) in a series of studies, effectively abrogated the irradiation enhanced IgE response of low and intermediate responder mice by passive transfer of FCA immune serum obtained from syngeneic donor mice. Moreover, adoptive secondary IgE responses of recipients of primed syngeneic spleen cells i.e. in the recipients mounting a high IgE response were totally abolished by passive transfer of FCA immune serum or ascitic fluid from FCA-immune mice. The suppressive activity of FCA immune serum was diminished or eliminated by exposure of FCA-primed donor mice to low dose of irradiation at an appropriate point during the priming regimen. Low doses of irradiation were not effective in eliminating suppressive activity of FCA-induced ascites fluid obtained from donor mice inoculated repeatedly with FCA. In contrast to the capacity of FCA immune serum from isologous donors to abrogate irradiation enhanced IgE responses of low responder mice, similar sera or ascitic fluids were ineffective in diminishing irradiation enhanced responses of high responder mice. These results indicated that the suppressor mechanism varies among different inbred mouse strains without any relationship to H-2 haplotype since even high responder mice did not produce significantly higher levels of IgE antibodies after elimination of the nonspecific suppressor mechanism (111,112). Therefore, the low response produced by some strains of mice was not the expression of a genetic inability of these strains to respond but rather a genetic capability to actively suppress IgE antibody production. These authors believe that

FCA immune serum induced in low responder mice was associated with the Ts cells previously described (p.17) since irradiation of mice before the induction of these molecules by FCA eliminated the suppressive effect. In contrast, mice inoculated with FCA and irradiated on the same day produced the suppressive molecules. These results, therefore suggested that the irradiation eliminated the cells producing the molecules.

The FCA immune serum was strain specific and in studies with selected mouse strains congenic at the H-2 histocompatibility complex, a role, at least in part, of one or more H-2 linked genes in dictating the strain specificity of such activity was demonstrated (113). The role of various lymphoid cells in the production and/or activity of these molecules was demonstrated also. Diminution of macrophage activity in situ increased the suppressive activity of sera from both FCA-immune and nonimmune low responder mice. Furthermore, the suppressive activity was absorbed by spleen cells from mice of the appropriate H-2 haplotype.

An enhancing factor was produced also by mice injected with FCA. Conclusive evidence was obtained in studies performed with lectin chromatography. The enhancing factor bound very strongly to oncanavalin-A Sepharose columns whereas the suppressor factor did not. The conditions for optimal production of the enhancing factor, (i.e. the dose of irradiation and FCA treatment) was slightly modified from those for the induction of suppressive factor. Nevertheless, two serum molecules with opposite biologic functions were produced (114).

Katz and his colleagues (114) postulated a new concept to explain the differences between individuals manifesting nonallergic and allergic phenotypes. This concept, termed "allergic breakthrough", explained that one of the avenues toward the allergic phenotype involved

coincidental sensitization combined with an imbalance in the normal dampening mechanism that served to limit IgE production. The authors demonstrated that i) certain manipulations effectively heightened or re-established a persistent dampening mechanism in the production of specific IgE antibodies; ii) once the allergic breakthrough had occurred, the production of IgE antibodies specific for the sensitizing antigen remained elevated at levels characteristic of the allergic phenotype, even after the threshold of the dampening activity returned to normal levels; iii) the allergic breakthrough displayed specificity, in that responsiveness occurred with subsequent sensitization to the specific antigen but not for other unrelated antigens.

The specific abrogation of the IgE antibody response to an antigen was achieved and stemmed from work performed in other classes of antibody. Havas (115) established a tolerant state in animals treated with a hapten-carrier conjugate with low immunogenicity. In contrast, an immune response was induced against the carrier and hapten if the carrier was itself immunogenic.

Golan and Borel (116), and Hamilton and Miller (117), produced a state of unresponsiveness to DNP, by injecting either conjugates of DNP and isologous gamma globulin or conjugates of haptens and syngeneic erythrocytes. The primary and secondary responses of the animals treated with hapten-syngeneic erythrocytes were suppressed, and by employing an adoptive cell transfer system, the researchers concluded that the unresponsive state was the result of B cell tolerance (118) and not T cell tolerance. Similarly B cell tolerance was mainly induced in mice injected with hapten-isologous gamma globulin (116). Moreover, a hapten-specific B cell tolerance was achieved also in mice treated with the nonimmunogenic

DNP-conjugate of D-GL (119-121).

Katz et al (121) was the first to report the abrogation of anti-hapten IgE response by the use of haptens conjugated to nonimmunogenic synthetic carriers. Conjugates of DNP with D-GL induced a hapten-specific unresponsive state in the IgE B cell population of normal and of primed mice. This unresponsive state was not reversed by treating the cells in vitro with trypsin (119) indicating that the responsiveness of the cells specific for DNP determinants was irreversibly inhibited.

Lee and Sehon (reviewed in 6 and 128) examined the effects of several conjugates of haptens with nonimmunogenic carriers on the anti-hapten IgE responses. Thus, mice treated with hapten-isologous gamma globulin (MγG) conjugates effectively suppressed the anti-hapten IgE response. The initial work was performed with DNP-MγG (3,4,123,124) which prevented the development of the primary anti-hapten IgE response in mice, but more importantly abrogated the ongoing IgE response. The unresponsive state of these animals was highly hapten-specific and maintained for an extended period of time (experimentally determined up to 8 months) by repeated injections of the conjugate at intervals of two months.

Employing the same system, cell transfer experiments were performed as to determine the nature of the suppression. The possibility of neutralization of the antibody by the conjugate did not explain the long term suppressed state induced in the mice, as the conjugates were cleared from the animals in approximately thirty days. Furthermore, the spleen cells, from animals tolerized with DNP-MγG 51 days prior to adoptive transfer into irradiated animals remained unresponsive in these

recipients even after a sensitizing dose of antigen was administered. However, the tolerant state was not transferred by serum of animals, which had been treated with DNP-MγG 20 days prior to bleeding, indicating that the conjugate had been cleared from the sera of these mice. The unresponsive state of the mice was not maintained, however, with injection of normal or primed cells into suppressed animals, but rather these mice responded according to the type of cell they had received (i.e. either a primary or secondary response was exhibited). Similarly, an admixture of treated and primed cells administered into irradiated recipients resulted in the induction of a secondary IgE response. Moreover, the suppressive state was maintained in two successive transfers into irradiated recipients even upon repeated exposure to antigenic stimulation.

Furthermore, Lee and Sehon provided evidence that particularly implicated the inactivation or elimination of the B_{ε}-cell population as the cause of the unresponsive state (6). It was shown that (i) the IgE response was elicited with a mixture of thymus cells of mice treated with the conjugate and bone marrow cells of primed mice, but not by a mixture of thymus cells of primed mice and bone marrow cells of suppressed mice; (ii) the ability of spleen cells, from mice which had been treated with DNP-MγG to produce IgE antibodies was restored when supplemented with bone marrow cells of primed mice; (iii) cooperation of splenic T cells of mice treated with the conjugate with splenic B cells of primed mice resulted in a marked IgE response, but the mixture of primed T cells and suppressed B cells produced only a minimal response and (iv) the ability of suppressed spleen cells to produce IgE antibodies was restored with anti-theta treated spleen cells of primed mice, but not with cortisone-resistant thymus cells of primed mice.

The studies of DNP-isologous gamma globulin was extended by Lee and Sehon in the examination of other haptens relevant to allergic diseases, i.e. penicillin (BPO) (123). Conjugates of MγG and penicillin G were successfully employed to establish an unresponsive state in mice. The results obtained in this system were virtually identical to those obtained with DNP-MγG as previously described. The suppressed state was established in normal and primed animals and was maintained in irradiated recipients with the transfer of tolerant spleen cells. Also, the tolerant state was specific for the hapten conjugated to the MγG since the response of the animals to the carrier was not affected.

Other nonimmunogenic carriers were used also to induce unresponsive states. Levan conjugates were employed by Desaymard and colleagues (125,126) to induce tolerance to various haptens. The levan, isolated from Corynebacterium levaniformis was conjugated to the hapten of interest and injected into mice. The treatment was found to abrogate the specific anti-hapten IgE response of these animals. B cell inactivation was shown to be the cause of the unresponsive state whereas T cells were not affected. Conjugates of Ficoll (M.W. 400,000) and hapten, specifically diminished the primary anti-hapten IgE response (127). However, suppression of the primary response was achieved only and no effect on the secondary response was detected. Furthermore, Diener and coworkers (127) employed the conjugate of a hapten and a nonimmunogenic carrier, carboxymethyl cellulose (CMC), for the induction of hapten-specific unresponsiveness. The anti-DNP IgE response of mice injected with DNP-CMC was specifically suppressed and was maintained also in irradiated syngeneic recipients, however suppressor T cells were not detected and it was concluded that the terminaton of the response was due to B cell inactivation.

Recently Lee et al (128,129,130) injected DNP or BPO conjugates of polyvinyl alcohol (PVA), a synthetic hydrophilic polymer (M.W. 10,000 or 14,000), into mice and suppressed the primary and secondary anti-hapten IgE and IgG responses. The anti-carrier antibody response was not affected by these treatments. The epitope density of the hapten on the PVA molecule played an important role in the establishment of the unresponsive state and in the allergenicity of the conjugates. Conjugates with epitope densities of less than 1.0 suppressed significantly the anti-hapten IgE response, but complete and long-lasting suppression of the reaginic response was not realized unless a conjugate of epitope density greater than 1.0 was employed. However, the conjugates with lower densities had lower allergenicity.

In addition, mice immunized with the sensitizing dose of antigen plus Bordetella pertussis, were prone to systemic anaphylaxis upon intravenous challenge with the multivalent hapten-protein conjugate. However, mice that received a tolerogenic dose of either DNP-PVA or BPO-PVA were protected from anaphylaxis induced by the appropriate hapten-protein conjugate. The protection afforded by these compounds was highly specific for the hapten, as evident from the fact that anaphylaxis occurred in the treated mice upon challenge with the carrier. As well, the unresponsive state was transferred into irradiated syngeneic recipients and was at least partially mediated by Ts cells.

The existence of carrier-specific suppressor T cells was demonstrated also in the regulation of IgE responses. Ishizaka and his coworkers (131) and Bach and Brashler (2) suppressed anti-carrier responses with urea-denatured (UD-C) or acetoacetylated carrier. The investigators injected, intraveneously, UD-C into animals primed with

the carrier and suppressed the response to the carrier, but also the antibody response to any hapten which had been attached to the carrier. The cellular mechanism was elucidated by injecting UD-C treated animals with spleen cells from mice primed with hapten-carrier. No increase of the IgE response was detected in mice treated with UD-C as compared to the controls. Also, the transfer of UD-C splenic T cells into normal nonirradiated mice greatly decreased the antibody response of the recipients. Thus, the suppression was mediated by Ts cells.

The Ts cells were specific for the carrier since a normal anti-hapten response was generated following immunization with the hapten coupled to an unrelated carrier. The effect of the Ts cells on a ongoing response was investigated by transferring splenic T cells from UD-C treated animals into mice displaying an IgE response to the hapten-carrier. The Ts cells depressed the ongoing antibody response, whereas splenic T cells from mice primed with carrier, failed to abrogate anti-carrier IgE responses. Thus, Ts cells suppressed not only the primary antibody response, but also the ongoing IgE antibody response (5). The suppressive effects of the UD-C treated splenic T cells was enhanced also by co-culturing the Ts cells with carrier or carrier bound to macrophage prior to transfer to irradiated recipients. These results supported the concept of macrophage involvement in the establishment of an unresponsive state induced by T cells (132,133).

Lee and Sehon (134,135) developed another system in which the primary and secondary antibody responses were specifically suppressed by conjugates of PEG with OA or RAG. The conjugates were devoid of the ability to combine with antibody induced to the unmodified antigen; either <u>in vivo</u> or <u>in vitro</u>. Thus, the conjugates neither elicited passive cutaneous anaphylaxis (PCA) reaction at sites sensitized with

murine IgE, nor caused systemic anaphylaxis in rats sensitized to the antigen nor combined with antibody to the native antigen to form precipitin bands. As well, the slow clearance of the conjugates as compared to that of native antigen, supported the evidence for non-immunogenicity of the PEG conjugates. The nonimmunogenicity of the conjugates and their tolerogenicity raised the possibility of their use in the clinical desensitization treatment of human patients.

Cell transfer experiments were performed in an attempt to identify the cellular mechanism(s), involved in the dampening of the IgE response by PEG conjugates. The state of unresponsiveness was maintained by transfer of spleen cells from mice treated with PEG conjugates into irradiated recipients. The spleen cells of suppressed mice when transferred into normal animals, suppressed the anti-carrier and anti-hapten IgE responses following sensitization with the hapten-carrier conjugate, indicating the presence of suppressor cells. Recently, these suppressor cells were characterized as T cells (personnal communication Dr. W.Y. Lee) since treating spleen cells from mice, previously administered PEG conjugates with anti-theta and complement, eliminated the suppressive capacity of the cells to dampen the IgE response of normal mice.

Liu and Katz (10) injected conjugates of protein antigens, such as OA or antigen E, with D-GL into mice and induced a state of long-lasting, antigen specific immunological suppression. The unresponsive state induced by the conjugates affected only the IgE class of antibody and specifically abrogated the response in unsensitized and presensitized animals. The injection of soluble antigen in the same dose as that in the conjugates led only to a transient depression of the IgE

response. The mechanism of the unresponsiveness obtained with antigen-D-GL conjugates did not involve the participation of active suppressor cells. The actual cellular mechanism has not yet been elucidated.

Another model of suppression of the IgE response was developed by Usui and Matuhasi (12). They demonstrated that conjugates of OA-pullulan (pullulan is a linear polymer of glucose), injected into mice induced a long-lasting depression of the IgE antibody response even upon subsequent immunization with OA. The suppression was antigen specific and was transferred into normal mice with spleen cells from donors treated with the conjugate 2 weeks prior to the transfer. The authors have speculated that the mechanism of the suppression involved either IgE B cell receptor blockade or possibly an active suppressor cell mechanism. No firm data was presented to substantiate the claims.

The induction of specific unresponsiveness of the IgE response by the treatment of mice with haptens or antigens covalently linked to nonimmunogenic carriers has been well established. However, the soluble form of the antigen when administered intraperitoneally or intraveneously has, at best, a transient effect on the IgE response. On the other hand, the administration of soluble or particulate antigens through the intragastric route has led to a marked and significant depression of the reaginic response (136,137,138).

David (13) demonstrated that rats fed with particulate antigen (SRBC) over a long period of time, became unresponsive to sensitizing doses of the antigen. Factors which were critical in the experiments were the age of the rats and the protocol used in the induction of the unresponsiveness. Thus, 2 or 5 month old rats mounted reaginic responses eventhough the rats were fed the particulate antigen.

In contrast 1 month old rats were rendered tolerant to the antigen. The animals had to be continuously fed since only with this protocol were the rats rendered refractory to the antigen. If feeding was stopped for 2 months, the recipients regained their ability to mount a humoral response to the antigen.

Vaz et al (137) demonstrated that a single large oral dose of antigen resulted in a profound inhibition of IgE and IgG_1 antibody formation after subsequent challenge of these mice with the antigen incorporated in aluminum hydroxide gel. Adoptively transferring normal or primed syngeneic spleen cells into the tolerized mice did not restore the immune response and the conclusion was drawn that the tolerance was maintained by an active suppressor mechanism. These results were confirmed by Ngan and Kind (138) who characterized the mechanism involved in the tolerance induced by feeding with antigen. The adoptive transfer of spleen cells or cells from Peyer's patches of mice which had been fed OA into normal syngeneic recipients inhibited the IgE and IgG_1 responses of the mice. Moreover, the treatment of the cells from OA fed mice anti-theta and complement, abrogated the capacity of these cells to inhibit the formation of IgE antibodies in the recipients. Furthermore, the Ts cells were antigen specific and the tolerance induced in the donors by antigen was also antigen specific. Cells of Peyer's patches from tolerant mice were more effective suppressors than spleen cells, since 10^7 cells from Peyer's patches rendered the recipients refractory to subsequent sensitizations whereas 10^8 spleen cells were needed to achieve the same effect. In addition, 20 mg of the antigen was required for the induction of splenic suppressor cells whereas 1 mg of antigen was sufficient to induce suppressors in Peyer's patches. The results suggested

that cells from Peyer's patches were the source of suppressor cells in spleen and lymph nodes after the oral administration of antigen.

The interpretation of these findings was supported by the results obtained by Mattingly and Waksman (139). The feeding of rats with SRBC induced specific suppressor cells within 2 days in Peyer's patches and mesenteric lymph nodes (MLN). After 4 days of feeding, suppressor cells were no longer found in the Peyer's patches or MLN but had appeared in the thymus and spleen. The possibility of circulating antigen suppressing the response was eliminated by employing radioactive antigen and looking for the specific activity in various organs. No detectable amount of antigen was found in the thymus or spleen. Thus, they concluded that suppressor cells originated in the Peyer's patches and/or MLN and subsequently migrated systemically into the spleen and thymus.

In the above studies by David, Vaz and Kind, tolerance induced by the intragastric route was successful when the animals used were unprimed, normal mice. Hanson et al (140) reported that the feeding of previously immunized animals with a single intragastric dose of 1 to 20 mg of OA, resulted in the production of secondary antibody response in these animals. The primary antigen binding titers (Farr assay) showed graded increases that were directly proportional to the oral dose of antigen. The time course of responses was parallel to responses in mice boosted i.p. with soluble antigen. The IgE antibody titers also increased significantly in response to OA feeding, but were not dose dependent. Despite effective boosting of ongoing antibody responses to parenteral immunization with OA in adjuvant the IgE antibody titers were significantly reduced in mice fed with larger doses of antigen. These results dissipate the possible use of antigen feeding for therapeutic purposes in man.

CHAPTER 2

SUPPRESSION OF THE IgE ANTIBODY RESPONSE TO OVALBUMIN IN MICE WITH A CONJUGATE OF OVALBUMIN AND ISOLOGOUS GAMMA-GLOBULINS

INTRODUCTION

In recent years hapten-specific tolerance has been induced in experimental animals by administration of conjugates of haptens (DNP, TNP, NIP, and BPO) linked to nonimmunogenic carriers, such as isologous serum proteins (3,4,115,116,141,142), isologous red blood cells and lymphocytes (143), nonimmunogenic or weakly immunogenic synthetic polypeptides (115,119,144) and polysaccharides (145). Furthermore, the immunosuppression due to conjugates of a hapten with the co-polymer D-GL (1,146) or with isologous γ-globulins (6) was shown to be attributable either to the irreversible inactivation or to receptor blockade of the hapten specific B cells.

The administration of tolerogens consisting of conjugates of haptens with isologous γ-globulins has proven effective in this and other laboratories for induction of immunologically specific suppression of the primary IgE antibody formation to DNP in the mouse (3,4,124), rat (147) and dog (148) and more importantly, for the marked suppression of an ongoing IgE response to DNP and BPO in sensitized mice (3,6,124). In addition to being hapten-specific, this immunosuppression was long-lasting, the state of unresponsiveness being maintained for an extended period of at least eight months, by injections of the tolerogen at intervals of two months (3).

More recently, specific suppression of IgE antibodies has been achieved with allergens modified by denaturation (131) photooxidation (149) or coupling to PEG (134,135) or D-GL (10). In all these systems

- with the possible exception of D-GL modified allergens - the participation of specific suppressor cells was shown to be responsible for the dampening of the IgE response by these modified allergen preparations.

Although the induction of hapten-specific tolerance with hapten conjugates of isologous γ-globulins is well documented, the possibility of using murine γ-globulins (MγG) for the synthesis of tolerogenic derivatives of polymeric molecules, such as protein antigens or pollen allergens, has not been reported. Hence, this investigation was initiated with a view to determining whether or not coupling of a protein antigen to MγG with the aid of glutaraldehyde could lead to the conversion of the antigen into a tolerogenic derivative which would be capable of inducing specific suppression of the immune response in inbred mice to the antigenic determinants of the unmodified antigen. Ovalbumin (OA) was selected as a model allergen for the present study, since it is readily available in pure form, its molecular weight is in the range of molecular weights of protein allergens present in pollen extracts and it represents also an allergen found in a variety of human food preparations.

The results of this investigation demonstrate that administration of OA-MγG conjugates into normal mice or into mice presensitized with DNP_3-OA suppressed, respectively, the capacity of these mice to mount a primary or secondary IgE response to both OA and DNP on further immunization with DNP_3-OA. However, presentation of the hapten (i.e. DNP) on a different carrier (i.e. ASC =Ascaris suum) to mice pretreated with OA-MγG led to normal anti-DNP and anti-ASC IgE responses. Furthermore, it was shown that the suppression induced by OA-MγG was not reversed by the transfer of spleen cells from normal or DNP-OA primed animals, i.e.

the recipients of cells remained immunosuppressed even on further immunization with a sensitizing dose of DNP-OA. It was also shown that transfer of spleen cells from tolerized mice into normal or immunized syngeneic recipients resulted in the suppression of IgE responses of the recipients on further sensitization with DNP-OA. On the basis of all these findings it was concluded that the suppression induced by OA-MγG conjugates was mediated by OA-specific suppressor cells.

MATERIALS

ANTIGENS

Ovalbumin (OA), ribonuclease (RNase) and bovine serum albumin (BSA) were purchased from Nutrional Biochemicals, Cleveland, Ohio. Ascaris suum extract (Asc), dinitrophenylated Ascaris suum extract (DNP-Asc) dinitrophenylated normal mouse serum (DNP-NMS) and dinitrophenylated-bovine serum albumin (DNP_{17}-BSA) were prepared by Dr. W.Y. Lee as previously described (3).

The conjugate, DNP-OA was prepared by reacting 200 mg of OA with 150 mg of the sodium salt of 2,4 dinitrophenylsulfonic acid in a total volume of 12 ml of 0.2 M Na_2CO_3 at room temperature for 3 hours. The uncoupled hapten was removed by gel filtration through a column (2.5 x 50 cm) of Sephadex G-25 equilibrated with a solution of phosphate-buffered saline (PBS) containing 0.15 M NaCl in 0.02 M phosphate buffer, pH 7.2. For the calculation of the average number of DNP groups in the conjugate the average molecular weight of ovalbumin was taken as 45,000 daltons.

OTHER REAGENTS

Freund's complete adjuvant (FCA) was purchased from Difco, Detroit Michigan, U.S.A. Sheep red blood cells (SRBC) in Alsever's solution and normal mouse serum (NMS) of $B_6D_2F_1$ were obtained from North American Laboratory Supplies, Gunton, Manitoba. Sephadex G-200 gel and the glass column (2.6 x 100 cm) were purchased from Pharmacia, Uppsala, Sweden. Micro hemagglutination apparatus, ammonium sulfate, boric acid, monobasic potassium phosphate, dibasic sodium phosphate and sodium chloride were purchased from Fisher Scientific Co. Ltd., (Montreal, Quebec), glutaraldehyde from Eastman Kodak, (Rochester, N.Y.), and ^{125}I in the form of NaI from Amersham/Searle Cooperation (Arlington

Heights Illinois, U.S.A.) respectively.

ANIMALS

For immunization, 6 to 8-week old (C_{57}BL/6 x DBA/2) F_1 mice (designated at $B_6D_2F_1$) of either sex were purchased from North American Laboratory Supplies, Gunton, Manitoba and Jackson Laboratories, Mass., U.S.A. For the passive cutaneous anaphalaxis (PCA) assays, random bred male hooded rats were obtained from Canadian Breeders Laboratory Supplies, Montreal, Quebec and North American Laboratory Supplies, Gunton, Manitoba. Precipitating anti-OA and anti-normal mouse serum (anti-NMS) antibodies were produced in New Zealand white rabbits purchased from Bio Laboratories, (St. Paul, Minnesota).

IMMUNIZATION

For the induction of the optimal IgE responses, mice were injected intraperitoneally (i.p.) with 1 μg of DNP_3-OA in the presence of 1 mg of freshly prepared aluminum hydroxide gel in 0.5 ml of saline as recommended by Levine and Vaz (72) - reproduced in this laboratory - (3). For the sake of brevity, this immunizing dose will be referred to hereafter as the sensitizing dose of DNP-OA. Similarly, 10 μg of DNP-Asc or RNase, incorporated with 1 mg of $Al(OH)_3$ in 0.5 ml of saline, were used for the induction of the corresponding reaginic antibodies and these mixtures will be referred to as the sensitizing dose of the appropriate antigens.

In all experiments, unless otherwise specified, the mice within a given group (four to five mice per group), which had received an identical treatment, were bled from the tail vein at different intervals after immunization and their sera were pooled for the measurement of antibody levels. All experiments reported in this study have been performed at least twice.

For the induction of precipitating anti-OA or anti-NMS antibodies, rabbits received, once a month for three consecutive months, an intramuscular injection of 1 mg of OA or NMS emulsified in FCA and were bled seven days after the third injection. Further injections of these rabbits with 1 mg of OA or NMS, emulsified in FCA, were given on alternate weeks with bleedings on each subsequent week. The serum samples collected from each rabbit were pooled and precipitated at $4^{o}C$ with ammonium sulfate at 50% saturation. The precipitate was then collected by centrifugation in a Sorvall RC-3 centrifuge (Newton, Connecticut, U.S.A.) at 39,000 x g for 20 minutes, dissolved in distilled water corresponding to the original volume of the serum, and dialyzed extensively against phosphate buffered saline, pH 7.2 (PBS). The precipitate dissolved readily in water and no insoluble residue of euglobulins was detected probably because of the high concentration of ammonium sulfate in the precipitate.

MEASUREMENT OF SERUM IgE ANTIBODIES

The IgE levels in mouse serum were determined by PCA in random bred hooded rats. The rats were closely shaven and, for sensitization of the shaven areas of the skin, volumes of 50 µl of serum, in two or four-fold serial dilutions, were injected intradermally into 2 rats and challenged 24 hours later by an intravenous (i.v.) injection of 1 mg of DNP-NMS, OA, RNase or Asc in 1 ml of saline containing 0.5% Evans blue dye.

For comparison of the reaginic antibody titers of different pools of sera from groups of four to five mice, the end point of the titration was taken as the reciprocal of the highest dilution of each serum resulting in a reaction of 5mm in diameter. The PCA titers for

one and the same reaginic serum determined in different rats were reproducible within a factor of 2; all PCA titers are reported as averages of two determinations.

MEASUREMENT OF HEMAGGLUTINATING ANTIBODIES

The presence of other classes of antibodies in sera of immunized mice was demonstrated by the passive hemagglutination procedure, utilizing conjugates of SRBC with DNP_{17}-BSA or OA prepared with the aid of glutaraldehyde as described by Avrameas (150). For this purpose, SRBC which had been maintained in Alsever's solution were washed three times by gentle centrifugation through PBS. To a solution of DNP_{17}-BSA or OA (40 mg in 20 ml) was added 1 ml of the packed red cells followed by dropwise addition of 2.5 ml of a freshly prepared 2.5% solution of glutaraldehyde with constant stirring. The reaction was allowed to proceed at room temperature for one hour. After washing the cells three times with PBS a stock suspension was prepared by suspending 1 ml packed red cells in 4 ml of PBS.

The assay was performed in micro hemagglutination plates using the Microtiter apparatus (Cooke Engineering Co., Alexandria Virginia). Briefly, volumes of 25 µl of normal rabbit serum, which had been hundredfold diluted with PBS (1% NRS), were dispersed into each well of the plate with the aid of the microdiluter; 25 µl of the serum to be tested was then diluted serially in the plate. The suspension of sheep erythrocytes (25 µl) coated with either OA or DNP_{17}-BSA, which had been diluted 14 times from the stock with 1% NRS, was then added to each well. The plate was then shaken gently and incubated at $37^{o}C$ for 1 hour. The hemagglutination titer was determined as the reciprocal of the highest dilution of an antiserum at which the agglutination was still barely detectable.

To ascertain that the test was specific and that the sensitivity

was not vitiated by errors during conjugation of the antigen to the erythrocytes, two control sera were used for each plate; (a) normal mouse serum was serially diluted instead of the test serum (this control was used to establish that the cells were not aggregated merely by cross-linking with glutaraldehyde or by some nonspecific factor) and (b) rabbit or mouse anti-serum containing anti-OA or anti-DNP antibodies, which had also been previously standardized by Dr. W.Y. Lee, was serially diluted in the wells of the microplates, (this test served as a positive control to ascertain the sensitivity of the method). When the results of these control tests were not satisfactory, the hemagglutination assay was repeated for all the sera in the same plate.

ADOPTIVE CELL TRANSFER

In the adoptive cell transfer experiment, spleens were removed from donor mice at different intervals after sensitization and/or treatment, as indicated for the appropriate experiments. Single cell suspensions in Eagle's minimum essential medium (MEM) (Flow Laboratories Inc., Rockville, MD), buffered with 20 mM hepes (N-2-hydroxyethylpiperazine-N' - 2 ethanesulfonic acid, Calbiochem, San Diego, Calif.) were prepared, washed and transferred i.p. into syngeneic recipients (3). The recipient mice were then administered the sensitizing dose of the antigen.

PREPARATION OF OA-MγG CONJUGATE

The conjugate, OA-MγG was prepared with the aid of glutaraldehyde using a modified version of the procedure developed by Avrameas (149). First of all for the isolation of mouse gamma-globulins, NMS was treated with ammonium sulfate at 50% saturation at $4^{\circ}C$. The precipitate was then collected by centrifugation in a Sorval centrifuge using the SS-34 head at 18,000 RPM for 20 minutes. The supernatant was decanted and the precipitate was dissolved in distilled water corresponding to the original

volume of the sera. After extensive dialysis against borate buffered saline, pH 8.0 (BBS), the solution was then fractionaed by gel filtration through a Sephadex G-200 column (2.6 x 100 cm) which had been equilibrated with BBS.

The elution profile appeared as three major peaks and the fractions in the second peak, corresponding to the elution volume of IgG referred to hereafter as mouse γG (MγG) were pooled and concentrated and re-fractionated on the Sephadex G-200 column to minimize contamination with IgM and serum albumin. As will be shown on page 49, this preparation of IgG contained trace amounts of IgM and serum albumin. However, it was considered to be sufficiently pure for the intended purpose and was used without further purification.

For the preparation of the tolerogenic conjugate of OA with MγG, 20 mg of OA in 4 ml of BBS was mixed with 20 mg of MγG in 4 ml of BBS. To this mixture was added dropwise, and with stirring, 200 µl of a 1% solution of glutaraldehyde (150). The reaction mixture was maintained for two additional hours at room temperature and then dialyzed extensively for 24 hours, against BBS for elimination of any free glutaraldehyde. The resulting products were purified by gel filtration through a Sephadex G-200 column (2.6 x 100 cm) using BBS as the eluting buffer.

ISOLATION AND CHARACTERIZATION OF OA-MγG CONJUGATE

1. Fractionation Studies:

The OA-MγG preparations were subjected to gel filtration through Sephadex G-200 columns (2.6 x 100 cm) which had been equilibrated with BBS. The different fractions were then analyzed by immunodiffusion and immunoelectrophoresis procedures for the presence of OA and MγG.

2. Immunodiffusion Analysis:

The immunodiffusion analysis was performed on microscope slides by an adaptation of the Ouchterlony technique (151) using a 1% agar gel, (Noble agar, Difco, Michigan) in veronal buffer, pH 8.2.

3. Immunoelectrophoresis:

Immunoelectrophoresis was performed in 1% agar gel in veronal buffer, pH 8.2 in a Deluxe electrophoresis chamber, (Gelman Instrument Company,) for 1 hour at 400 volts.

4. Determination of the ratio of OA to MγG:

To determine the molar ratio of OA to MγG in the conjugate, the MγG was trace-labelled with ^{125}I using the chloramine T method (152). Thus, to 1 mg of MγG in 0.5 ml of PBS was added 1 mCi of Na ^{125}I followed by 0.2 ml of freshly prepared chloramine T reagent (0.1 mg in 1 ml PBS). The reaction was allowed to proceed for 5 minutes at room temperature with frequent mixing. To stop the reaction, sodium metabisulfite ($Na_2S_2O_3$) (0.2ml) was added to the mixture which was then fractionated through a Sephadex G-25 column (1.5 x 15 cm) using BBS as eluting fluid. The fraction appearing in the void volume was collected and dialyzed overnight against BBS.

The solution was concentrated under reduced pressure, and the protein concerntration was determined spectrophotometrically with the Carl Zeiss PMQ3 spectrophotometer using an extinction coefficient of 14 for a solution containing 10 mg of globulins per ml at 280 nm (153). The percentage of ^{125}I bound to MγG was determined by the following method. Equal volumes of the labelled protein were added to two 5-ml Sorvall centrifuge tubes and were adjusted to 0.5 ml with a solution of PBS contained 0.05% BSA. To one tube was added 0.5 ml of a 20% trichlo-

roacetic acid (TCA) and to the other 0.5 ml of PBS. The tubes were then stirred, cooled to $4^{o}C$ and centrifuged at 15,000 RPM for 10 minutes and the level of radioactivity of the supernatant in each tube was determined in a Beckman Gamma Counter, model number γ-300. The fraction of ^{125}I bound was calculated with the aid of the formula:

$$\%^{125}I \text{ bound} = 100 - (100 \times \frac{\text{CPM in tube with TCA}}{\text{CPM in tube with PBS}})$$

In all experiments at least 95% of the ^{125}I was associated with the protein precipitated with TCA.

Unlabelled MγG was added to adjust the protein concentration to 20 mg per 4 ml and the specific activity of the trace-labelled MγG was determined for a small portion (50 µl) of the MγG solution which had been diluted with PBS. The OA was coupled to the ^{125}I-labelled MγG as previously described. The reaction mixture was filtered through the Sephadex G-200 column and the concentration of each fraction was determined spectrophotometically in terms of its optical density and its radioactivity was determined in the Beckman Gamma Counter.

5. The "in vivo" half-life of the OA-MγG conjugate:

The half-life of the conjugate was estimated by determining the rate of clearance of 600 µg of ^{125}I-labelled OA-MγG which had been administered i.v. into normal mice. Sodium iodide (0.1%) was added to the drinking water of the mice so as to minimize thyroidal uptake of the radioactive iodide (154). Mice were bled from the retroortibal sinus at different intervals and the radioactivity associated with 25 µl of each blood sample was determined in the Beckman Gamma Counter. After the level of radioactivity in the serum approached the background levels, the mice were sacrificed and the radioactivity levels in the liver, spleen,

kidney, thymus and heart were determined.

6. Determination of Molecular Size:

The molecular size of the fraction considered to consist of OA-MγG was determined in terms of its elution volume from a column of Sepharose-4B (1.25 x 100 cm) equilibrated with BBS; the filtration properties of which had been standardized with Blue Dextran 2000, mouse IgM, mouse IgG and mouse serum albumin.

7. Preparation of OA-OA and OA-RγG:

The polymerization of OA with the aid of glutaraldehyde was carried out as follows: To a solution of 40 mg of OA in 8 ml of BBS was added dropwise, with constant stirring, 0.4 ml of a 1% solution of glutaraldehyde. The mixture was allowed to react for 2 hours at room temperature and dialyzed for 24 hours at $4^{\circ}C$ against BBS. The reaction products were then fractionated by gel filtration through a Sephadex G-200 column (2.6 x 100 cm) equilibrated with BBS.

The conjugation of OA to rabbit IgG (RγG) was performed as described for the coupling of OA with MγG with the aid of glutaraldehyde.

RESULTS

A) EVIDENCE FOR THE PREPARATION OF OA-MγG CONJUGATE

The reaction mixture containing the OA-MγG conjugates prepared by the procedure described under "Methods" was fractionated by gel filtration through a Sephadex G-200 column. As can be seen from the elution profile illustrated in Figure 1, there were two distinct peaks, the first emerging in the void volume of the column and the second in the elution volume corresponding to that of free ovalbumin. No distinct fraction emerged in the elution volume corresponding to that of MγG, which may be interpreted as indicating that MγG had been incorporated into high molecular weight polymers by the reaction with OA or by polymerization of MγG itself in the presence of glutaraldehyde. However, the latter possibility did not appear to be true as evident from the results in immunoelectrophoresis analysis as described later.

Analysis of the sample in the first peak by immunodiffusion and immunoelectrophoresis, with the aid of rabbit antisera to OA and to NMS, revealed that it consisted of OA linked covalently to MγG. Thus, from the immunodiffusion pattern shown in Figure 2, it is clear that, whereas the antisera used were specific for their respective antigens (i.e. the anti-NMS and anti-OA sera did not crossreact with OA and NMS respectively), the conjugate contained the antigenic determinants of both OA and MγG and that these were present on the same molecular moiety. The immunoelectrophoretic patterns of the conjugate with antisera to OA and NMS, illustrated in Figure 3, are essentially identical, confirming thus the previous conclusion that the conjugate isolated by gel filtration consisted primarily, if not exclusively, of molecular hybrids of both OA and MγG.

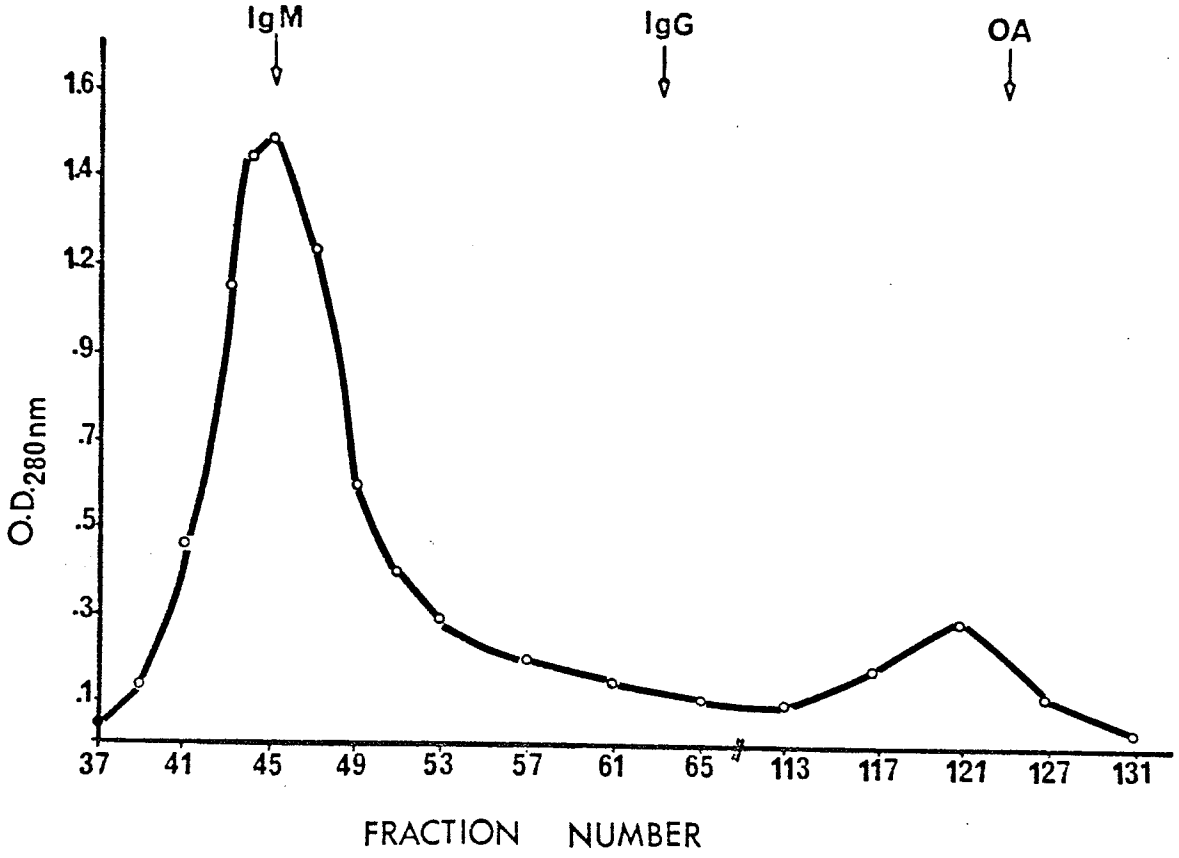


Figure 1: Elution Profile of OA-MγG through a Sephadex G-200 column

Ovalbumin was coupled to MγG with the aid of glutaraldehyde. After dialysis against BBS the reaction mixture was passed through a Sephadex G-200 column (2.6 x 100 cm) equilibrated with BBS, which had been standardized with respect to the elution volumes of mouse IgM, mouse IgG and OA, indicated by the arrows.

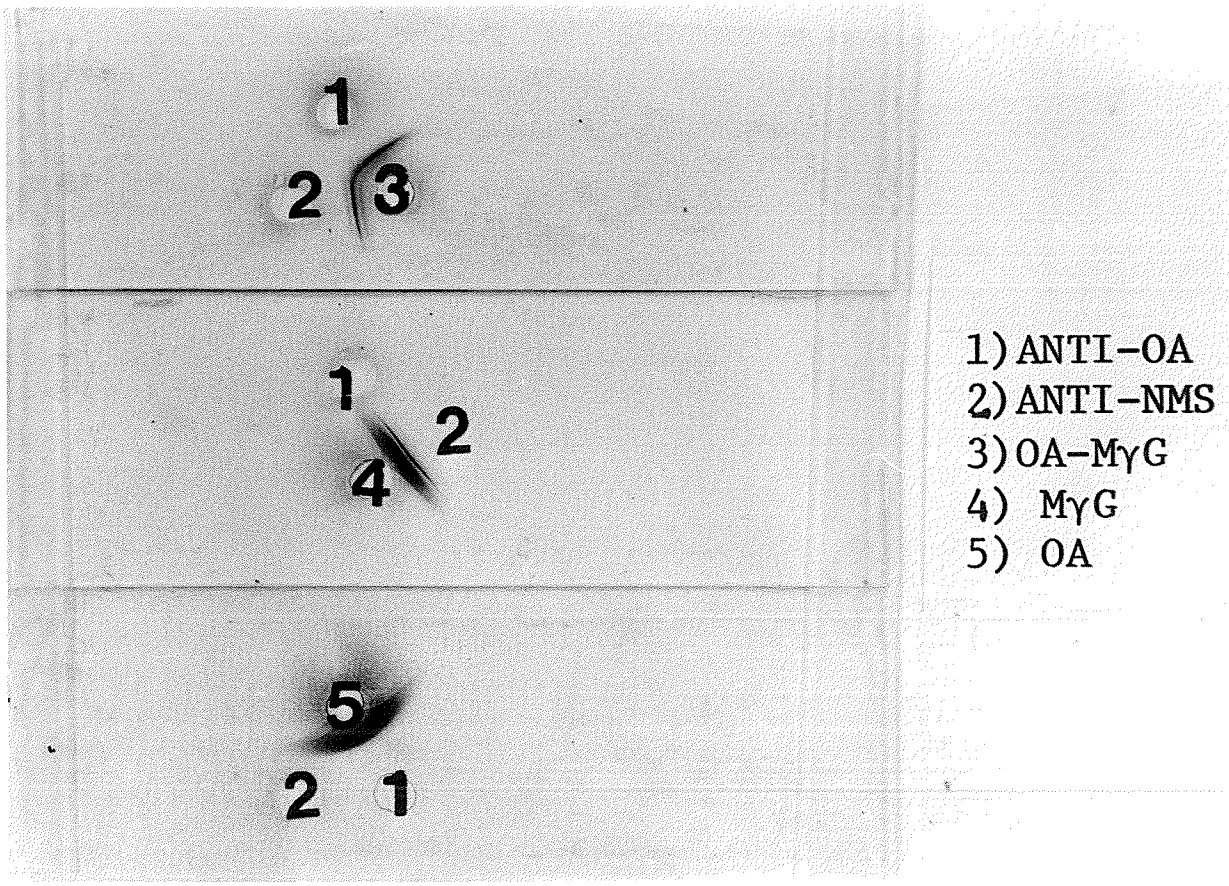


Figure 2: Immunodiffusion pattern of OA-MγG

Precipitin reactions of OA-MγG, OA and MγG with rabbit antisera to OA and NMS in agar gel.

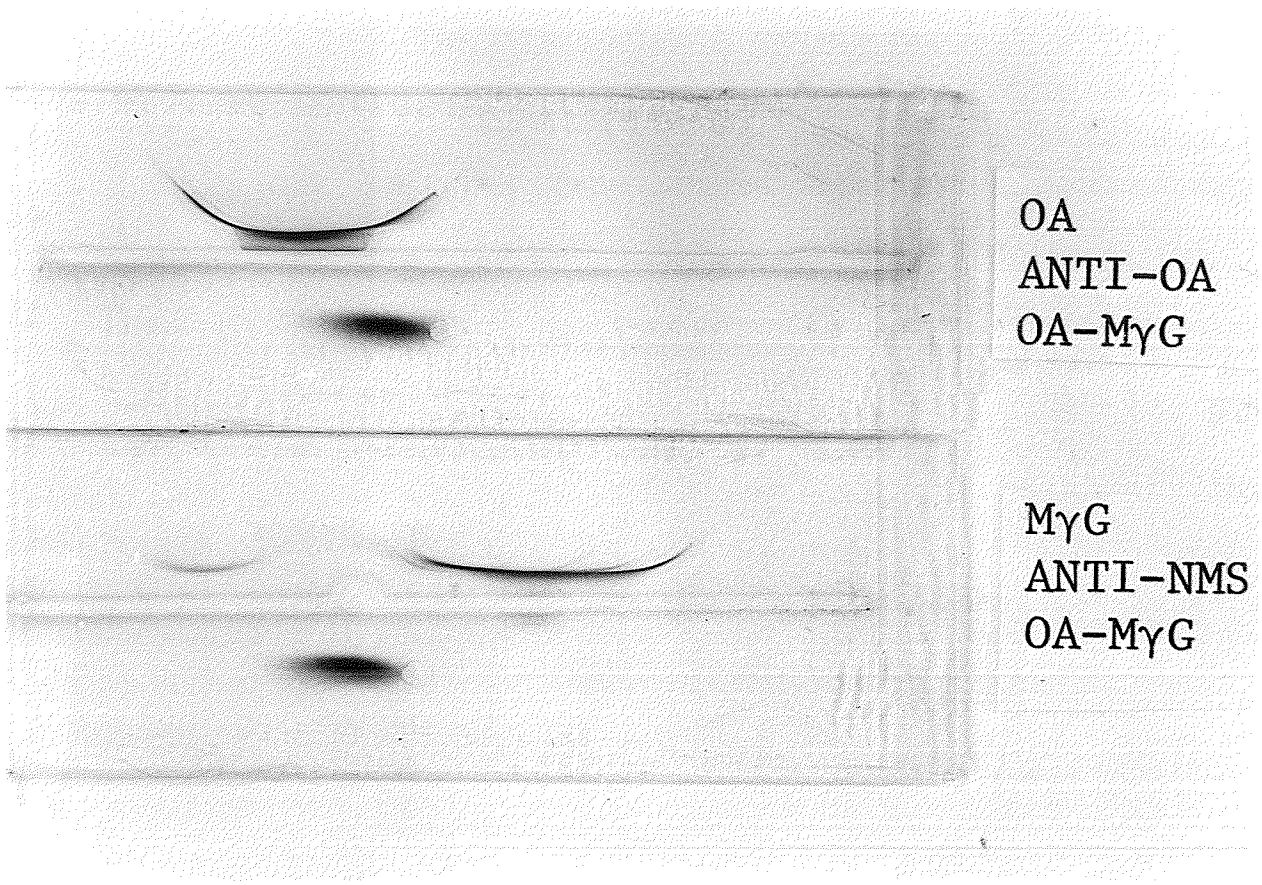


Figure 3: Immunoelectrophoresis pattern of OA-MγG

The OA-MγG conjugate was subjected to immunoelectrophoresis in agar gel in veronal buffer pH 8.2. The troughs, parallel to the axis in migration were filled with rabbit antiserum to either OA or NMS after electrophoresis together with two markers, OA and MγG which were used for comparison of the relative position of migration of the conjugate.

B) PHYSICOCHEMICAL CHARACTERISTICS OF OA-MγG CONJUGATE

To determine the molar composition of OA-MγG conjugate, MγG was radiolabelled with $Na^{125}I$ and then coupled to OA with the aid of glutaraldehyde as described earlier. This conjugate was then purified by filtration through a Sephadex G-200 column and the fractions were monitored in terms of their optical density at 280 nm and radioactivity in CPM. As is obvious from Figure 4, there was an overlap of the optical density and radioactivity profiles for the fractions which emerged in the void volume of the column, and which were described earlier to consist of OA-MγG conjugates. Moreover, as is evident from the radioacitvity profile in Figure 4, the ^{125}I-label was associated with two additional fractions whose elution volume corresponded to IgG and mouse serum albumin (MSA). Indeed the presence of M-IgG and MSA was demonstrated respectively in the fractions by immunodiffusion with the aid of rabbit antiserum to normal mouse serum. The latter was considered to represent the mouse serum albumin which had been shown to be present as a contaminant in the MγG preparation used for coupling with OA. Both the MIgG and MSA components were considered to be present only in minute amounts in these fractions since their contribution to the optical density profile was minimal. The fraction with an elution volume intermediate between these two components, (identified in Figure 4 as OA-OA) was shown by immunodiffusion to contain OA and was considered to consist of polymerized OA, OA_x-$M\gamma G_y$ and MγG.

The fractions designated in Figure 4 as OA-MγG were pooled and concentrated under reduced pressure. The molar ratio of OA to MγG in the conjugate was calculated as 6:1 in terms of the specific activities of MγG and OA-MγG and of the concentrations of these compounds which

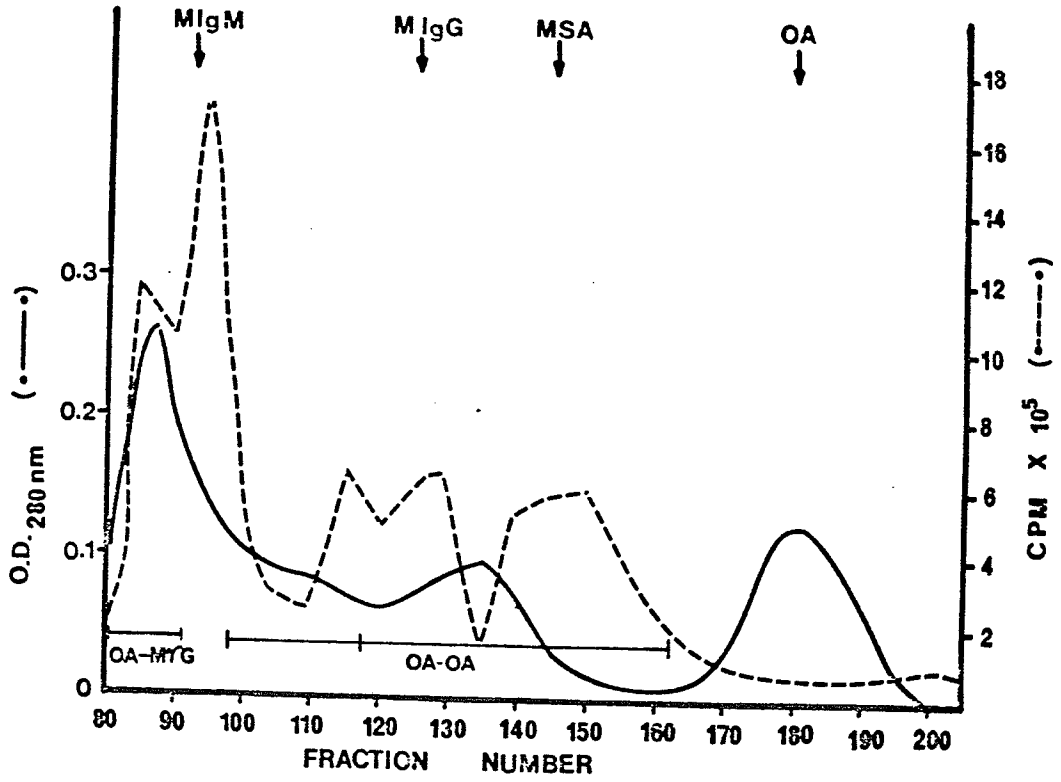


Figure 4: Elution profile of ^{125}I labelled MγG-OA conjugate

Ovalbumin was coupled to ^{125}I labelled MγG with the aid of glutaraldehyde and the conjugate was filtered through a Sephadex G-200 column (2.0 x 150 cm). Fractions of 3.2 ml were collected. The elution profiles of the emerging fractions were determined in terms of their optical density (o——o) and radioactivity (●·····●).

were determined as a function of their optical densities.

The molecular weight of OA-MγG was estimated from its elution volume with a column of Sepharose -4B which had been calibrated with Blue Dextran, mouse IgM and mouse IgG. On the basis of the elution profile of the OA-MγG conjugate preparation through this column, the eluates were arbitrarily pooled into three main fractions as plotted in Figure 5.

By immunodiffusion with the aid of rabbit antisera to NMS and OA, it was demonstrated that the conjugate emerging in the second fraction contained the antigenic determinants of both OA and MγG and that these were present on the same molecular moiety (please see Figure 2). Neither the first nor the third fractions gave a precipitin band with rabbit antiserum to NMS and OA respectively. Hence, the two fractions were disregarded and the second fraction containing the bulk of the OA-MγG conjugate was used for further characterization.

For further determinations of concentrations of OA-MγG conjugates from optical density measurements at 280 nm, an approximate extinction coefficient was calculated for the radioactively labelled conjugate with the aid of the expression given below, on the assumptions that (i) under identical coupling conditions the composition of the resulting conjugates was identical and (ii) the contributions of the extinction coefficient of OA and MγG in the conjugate were additive, i.e. that coupling of OA to MγG mediated by glutaraldehyde did not affect their individual extinction coefficients which were taken at 0.735 and 1.4 for solutions containing 1 mg/ml of the corresponding compounds.

$$E_{OA\text{-}M\gamma G} = \frac{E_{OA} \; W_{OA}^{conj} + E_{M\gamma G} \; W_{M\gamma G}^{conj}}{W_{OA\text{-}M\gamma G}}$$

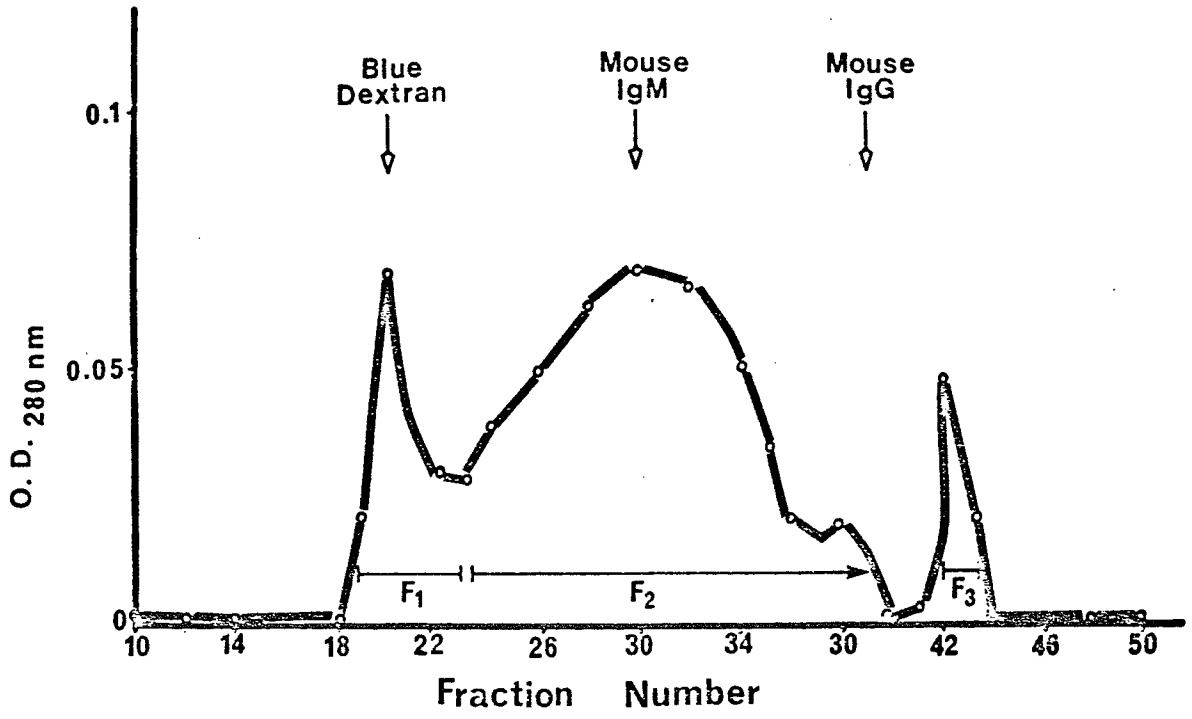


Figure 5: Elution profile of OA-MγG through a Sepharose 4-B column

The conjugate OA-MγG obtained from the Sephadex G-200 column (shown in Figure 4) was further fractionated by filtration through a Sepharose 4-B column (1.25 x 100 cm); the elution characteristics of which having been calibrated with Blue Dextran, mouse IgM and mouse IgG. The optical density of each fraction was measured.

In this expression, the terms E_{OA}, $E_{M\gamma G}$ and $E_{OA-M\gamma G}$, designate the extinction coefficients of OA, MγG and OA-MγG conjugate, respectively: the terms W_{OA}^{conj} and $W_{M\gamma G}^{conj}$ represent the weight fractions of OA and MγG in the OA-MγG conjugate and the term $W_{OA-M\gamma G}$ refers to the total weight of the conjugate.

C) RATE OF CLEARANCE OF OA-MγG IN NORMAL MICE

From the clearance data plotted in Figure 6, the half-life of OA-MγG in the circulation of normal mice was calculated as 3 days, which corresponds to the half-life for MγG reported by others (155,156). On the other hand, as shown in Figure 6, native OA had a much shorter half-life. Thus, only 0.1% of the injected ^{125}I-OA was present in the circulation 24 hours after injection and it was almost completely undetectable 6 days after administration. Consequently no attempt was made to determine the half-life for the clearance of ^{125}I-OA from circulation.

The distribution of ^{125}I-labelled OA-MγG conjugate, among different organs, 14 days after its administration is shown in Table 1. Thus, it appears that OA-MγG conjugate was preferentially taken up by the liver, kidney, and spleen and that as expected no significant amount of the conjugate was taken up by the thymus and heart.

D) PREPARATION OF OA-OA AND OA-RγG CONJUGATE

Ovalbumin was aggregated with the aid of glutaraldehyde as described earlier and the higher molecular weight products isolated by gel filtration through a Sephadex G-200 column were arbitrarily pooled into the three fractions F1, F2 and F3 (please see Figure 7). The elution volumes of these fractions correspond respectively to those of IgM, IgG and OA, and their molecular sizes were considered to be in the range of 10^6 and 1.6×10^5 and 4.5×10^4 daltons respectively. From the elution

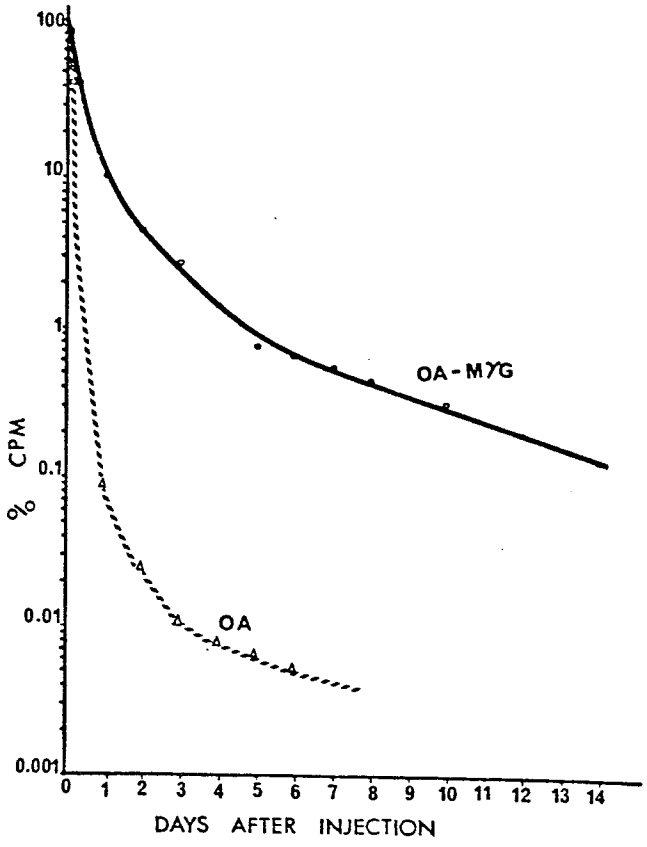


Figure 6: Determination of the clearance rate of ^{125}I labelled OA-MγG and OA in normal mice.

Each mouse received an i.p. injection of 600 µg ^{125}I labelled OA-MγG or OA. The mice were bled at regular intervals and the radioactivity of 25 µl of each sample of the whole blood was determined in a Beckman Gamma Counter.

TABLE 1

DISTRIBUTION OF ^{125}I LABELLED OA-MγG IN VARIOUS ORGANS

ORGAN	C.P.M.
LIVER	388,686±154.7
KIDNEY	61,234±137.45
SPLEEN	31,116±50.61
THYMUS	1,136±30.09
HEART	1,242±14.69

Mice received 600 µg of ^{125}I-labelled OA-MγG conjugate with an original specific activity of 1.61×10^7 C.P.M. After 14 days the mice were sacrificed and the total radioactivity in the above organs was determined.

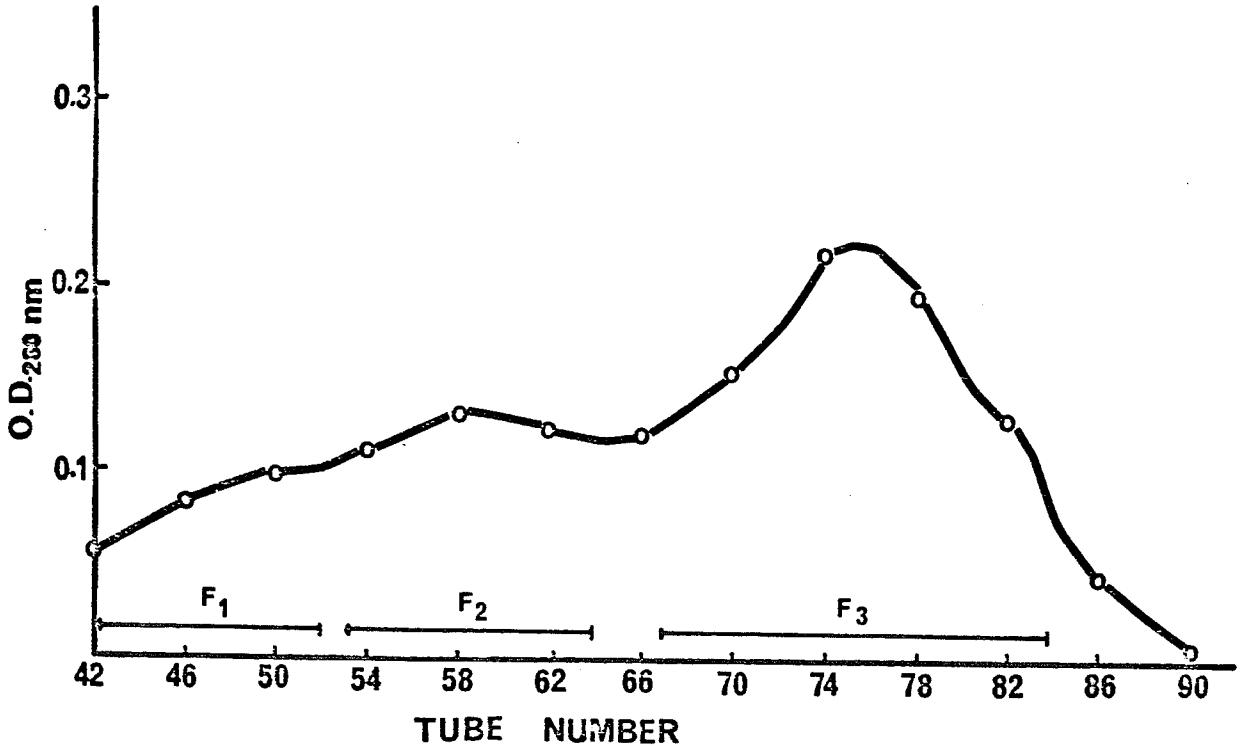


Figure 7: The elution profile of polymerized OA on gel filtration through a Sephadex G-200 column

Ovalbumin (40 mg in 8 ml BBS) was polymerized with the aid of glutaraldehyde (0.4 ml of 1% solution). After dialysis against BBS, the reaction mixture was passed through the Sephadex G-200 column (2.6 x 100 cm) which had been previously standardized with mouse IgM, IgG and OA. The individual fractions emerging from the column were arbitrarily pooled into fractions as indicated.

profile shown in Figure 7, it may be concluded that under the conditions used OA had been slightly polymerized with glutaraldehyde since approximately 80% of the OA emerged in the elution volume corresponding to that of native OA, i.e. fraction 3. The remaining 20% of the reaction products representing OA polymers of varying sizes emerged from the column in the elution volumes greater than that of fraction 3. The immunological properties of these fractions will be discussed later on.

The method for the preparation of OA-RγG conjugate was identical to that used for the conjugation of OA to MγG (as described on page 41), and the properties of the OA-RγG preparation were similar to those of OA-MγG, as judged from its elution profile from a Sephadex G-200 column and from its immunodiffusion and immunoelectrophoretic patterns, with the aid of a guinea pig antiserum to rabbit whole serum (which was used to detect rabbit IgG) and a rabbit anti-serum to OA. (The guinea pig antiserum was kindly donated by Mr. G. Lang). The immunological properties of OA-RγG will be discussed below.

E) IN VIVO EFFECTS OF OA-MγG

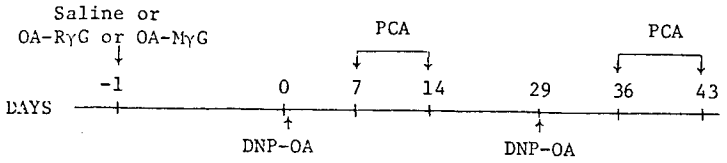
1. The effects of OA-RγG, OA-OA and OA-MγG conjugates on the primary response to DNP-OA.

(a) Effect of OA-RγG

Pretreatment of normal mice with the OA-RγG conjugate resulted only in a transient suppression of the primary anti-DNP and anti-OA responses (Test 2, Table II). However, upon reimmunization with the sensitizing dose of DNP-OA, these mice recovered their capacity to produce anti-DNP and anti-OA IgE responses. The kinetics of the secondary response was similar to that observed for the secondary response of control animals and by day 43 the magnitude of the response of animals pretreated with OA-RγG was indistinguishable

TABLE II

EFFECT OF ISOLOGOUS AND HETEROLOGOUS CARRIERS



GROUP	TREATMENT*		PCA TITERS		
	Day	Compound	Day	Anti-DNP	Anti-OA
Control	-1	Saline			
	0	DNP-OA	7	<10	<10
			14	640	2,500
	29	DNP-OA	36	5,000	2,560
			43	1,200	1,800
Test 1	-1	OA-MγG (475 µg)			
	0	DNP-OA	7	90	<10
			14	<10	<10
	29	DNP-OA	36	640	80
			43	200	160
Test 2	-1	OA-RγG (475 µg)			
	0	DNP-OA	7	<10	<10
			14	60	<10
	29	DNP-OA	36	1,200	900
			43	1,100	1,000

* All mice received sensitizing doses of DNP-OA on days 0 and 29. The animals in test groups received an i.p. injection of 475 µg OA-MγG (Test 1), or 475 µg OA-RγG (Test 2).

from that of the control group. By contrast, both the primary and secondary responses of the mice treated with OA-MγG were suppressed. These findings provide evidence for the capacity of conjugates of OA-MγG to inhibit the production of anti-OA IgE antibodies in mice for an extended period and that OA coupled to xenogeneic γ-globulins was not effective in inducing suppression of these antibodies.

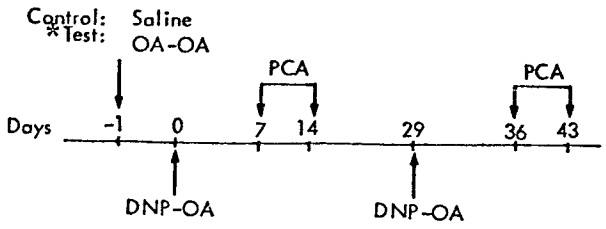
(b) Effect of polymerized OA.

Since one may visualize that polymers of OA may have been formed during the preparation of OA-MγG conjugate and that these polymers may have contributed to the observed immunosuppression, the following experiments were performed to test this possibility.

The tolerogenic capacity of fractions F1 and F2 (hereafter referred to as highly and moderatly polymerized OA fraction) was tested by administration into mice of single injections of various doses of each fraction ranging from 10-200 μg in 0.5 ml of saline per mouse, one day prior to immunization of the mice with the sensitizing dose of DNP-OA. From the results listed in Tables III and IV it would appear that the highly and moderately polymerized OA fractions had only a transient helper effect (rather than a suppressive effect) on the primary anti-OA IgE response detected on day 7. This effect waned away by day 14 and it is important to point out that these fractions had no effect on the secondary anti-OA IgE response induced by reimmunization of the mice with a second sensitizing dose on day 28. By contrast, both fractions had a slight depressive effect on the anti-DNP primary and secondary IgE responses. However, the hemagglutinating antibody response was not significantly affected by the fractions.

TABLE III

EFFECT OF HIGHLY POLYMERIZED OA ON THE PRIMARY ANTI-DNP AND ANTI-OA IgE RESPONSES

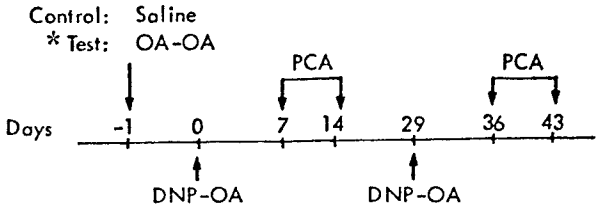


Group	Treatment		PCA Titers			Log_2 HA Titer	
	Day	Compound	Day	Anti-DNP	Anti-OA	Anti-DNP	Anti-OA
Control	-1	Saline					
	0	DNP-OA					
			7	<10	<10	3	1
			14	1625	1600	7	4
	29	DNP-OA					
			36	1350	1850	6	8
			43	1600	1900	6	9
Test 1	-1	OA-OA (10 µg)					
	0	DNP-OA					
			7	<10	880	2	2
			14	16	310	3	5
	29	DNP-OA					
			36	670	1850	6	10
			43	180	1500	5	10
Test 2	-1	OA-OA (100µg)					
	0	DNP-OA					
			7	<10	900	2	4
			14	<10	20	3	4
	29	DNP-OA					
			36	240	1800	6	10
			43	250	1900	5	10
Test 3	-1	OA-OA (200 µg)					
	0	DNP-OA					
			7	80	1600	2	4
			14	<10	<10	2	6
	29	DNP-OA					
			36	240	1650	4	10
			43	75	1800	6	11

* All test mice were administered varying doses of highly polymerized (Fl) OA followed by sensitizing doses of DNP-OA on days 0 and 29.

TABLE IV

EFFECT OF MODERATELY POLYMERIZED OA ON THE PRIMARY ANTI-DNP AND ANTI-OA IgE RESPONSES



Group	Treatment		PCA Titers			Log_2 HA Titers	
	Day	Compound	Day	Anti-DNP	Anti-OA	Anti-DNP	Anti-OA
Control	-1	Saline					
	0	DNP-OA					
			7	<10	<10	3	1
			14	1625	1600	7	4
	29	DNP-OA					
			36	1350	1850	6	8
			43	1600	1900	6	9
Test 1	-1	OA-OA (10 µg)					
	0	DNP-OA					
			7	300	310	5	1
			14	250	1450	5	5
	29	DNP-OA					
			36	670	1750	6	10
			43	700	1900	7	10
Test 2	-1	OA-OA (100 µg)					
	0	DNP-OA					
			7	80	1750	2	1
			14	<10	60	3	5
	29	DNP-OA					
			36	640	1750	6	11
			43	640	1800	6	11
Test 3	-1	OA-OA (200 µg)					
	0	DNP-OA					
			7	65	950	2	4
			14	<10	20	3	5
	29	DNP-OA					
			36	80	900	5	8
			43	230	1650	5	10

* All test mice were administered varying doses of moderately polymerized (F2) OA followed one and 29 days later by a sensitizing dose of DNP-OA

(c) Effect of OA-MγG

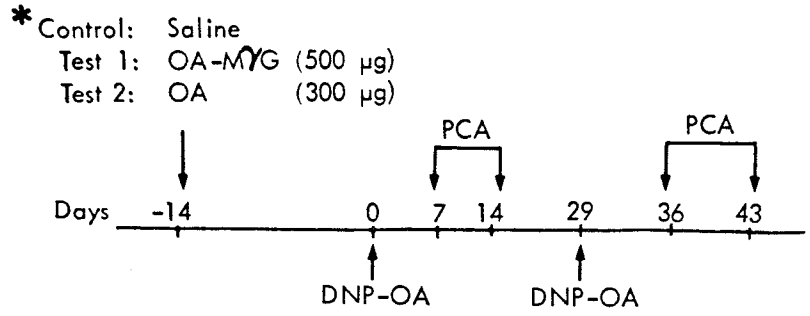
In other experiments the effect of varying the dose and time of administration of OA-MγG conjugate on its suppressive capacity was investigated. First of all, the effect of OA-MγG on the primary response of normal mice was investigated. A group of mice received an i.p. injection of 500 μg of OA-MγG per mouse, while two other groups of mice each served as controls; one of these received an i.p. injection of 300 μg of OA which was equivalent to the same amount of OA in 500 μg of OA-MγG and the other group received saline. All three groups received the sensitizing dose of DNP-OA 14 days later.

As is evident from the data listed in Table V both the anti-DNP and anti-OA responses were suppressed in mice pretreated with OA-MγG. Moreover, even upon reimmunization with the sensitizing dose of DNP-OA after a further interval of 29 days, the anti-DNP and anti-OA IgE response of these mice was 4 to 10 times lower than that of the control group which had been pretreated with saline. On the other hand, mice pretreated with OA mounted an enhanced anti-OA response on immunization with the sensitizing dose of DNP-OA. However, the anti-DNP response of the latter mice, although significantly higher than the anti-DNP response of mice in the former test group was partially suppressed in relation to the response of the control group.

The results of this experiment clearly indicate that an i.p. injection of OA-MγG did indeed suppress the immune response to OA and that the suppression was long-lasting. Moreover, it is clear from these results that i.p. pretreatment with OA proved not only ineffective in suppressing the IgE response, but did actually enhance the primary anti-OA IgE response.

TABLE V

EFFECT OF PRETREATMENT WITH OA AND OA-MγG ON THE PRIMARY ANTI-DNP AND ANTI-OA IgE RESPONSES



Group	Treatment		PCA Titers		
	Day	Compound	Day	Anti-OA	Anti-DNP
Control	-14	Saline			
	0	DNP-OA	7	<10	<10
			14	1100	3000
	29	DNP-OA	36	3050	4200
			43	4000	4000
Test 1	-14	OA-MγG			
	0	DNP-OA	7	300	<10
			14	260	80
	29	DNP-OA	36	640	1050
			43	950	1128
Test 2	-14	OA			
	0	DNP-OA	7	4000	320
			14	4000	320
	29	DNP-OA	36	5120	4200
			43	4000	5120

* All mice received sensitizing doses of DNP-OA on days 0 and 29. The animals in test groups received an additional i.p. injection of 500 µg of OA-MγG (Test 1) or 300 µg of OA (Test 2).

In the next experiment, the test and control mice received an i.p. injection of OA-MγG (700 µg/mouse) or of saline respectively. Both groups of mice received a sensitizing dose of DNP-OA, a few hours later and on day 29. The results (Figure 8) clearly demonstrate that the anti-OA IgE response was suppressed in the OA-MγG treated group and that the anti-OA IgE response was not significantly boosted by a second sensitizing dose given on day 29, whereas the control group demonstrated the typical anamnestic response. It is to be noted that the anti-DNP IgE response of the OA-MγG treated group was completely abrogated even upon resensitization with the sensitizing dose of DNP-OA. However, the hemagglutinating antibody response to DNP and OA was not significantly affected by the pretreatment of mice with OA-MγG (Figure 9), whereas the H.A. anti-DNP response was dampened. (An observation not consistently detected.)

To establish the effect of native OA on primary IgE response, using the experimental protocol above, OA (420 µg/mouse) was injected into mice and a second group received only saline. Both groups then received a sensitizing dose of DNP-OA within a few hours and a second dose on day 29. Mice, thus treated gave depressed primary anti-OA and anti-DNP IgE responses (Figure 10) but, upon resensitization on day 29 with DNP-OA the secondary anti-OA and anti-DNP IgE responses did not differ from those of control animals and persisted for an extended period. The results in Figures 8,9 and 10 demonstrate that OA-MγG is more effective than OA in inducing tolerance to OA.

2. Effect of OA-MγG on the secondary IgE responses to OA and DNP

In an attempt to establish if OA-MγG could depress an ongoing IgE response, an experiment was performed according to the protocol illustrated in Table VI. The mice were first primed with a sensitizing dose

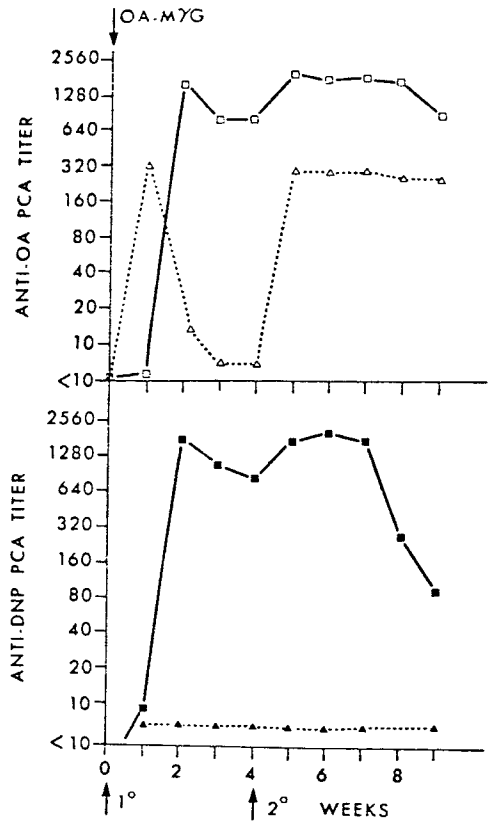


Figure 8: Effect of OA-MγG on the primary anti-OA and anti-DNP IgE responses

Mice were treated with 700 µg/mouse of OA-MγG or saline and sensitized within 4 hours with the sensitizing dose of DNP-OA. On day 29, both groups were resensitized with the sensitizing dose of DNP-OA. Open triangles (△·····△) and squares (□——□) represent respectively the anti-OA response of test and control groups while the closed triangles (▲- - -▲) and squares (■——■) represent respectively the anti-DNP response of test and control groups.

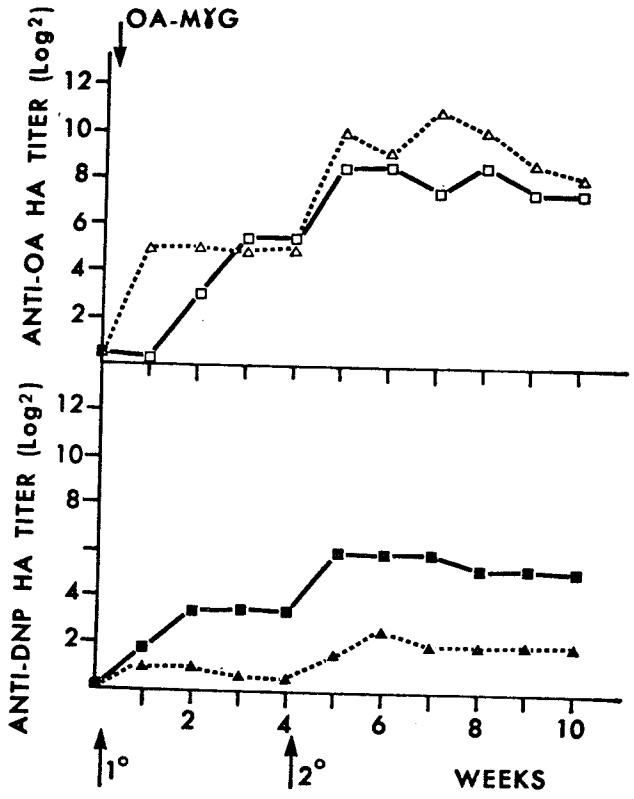


Figure 9: Effect of OA-MγG on the primary anti-OA and anti-DNP hemagglutinating antibody response.

Mice were treated with 700 µg/mouse of OA-MγG or saline and sensitized within 4 hours with the sensitizing dose of DNP-OA. On day 29 both groups were reimmunized with DNP-OA. Open triangles (△- - - -△) and squares (□——□) represent the anti-OA H.A. response for the control and test groups respectively, while the closed triangles (▲- - -▲) and squares (■——■) represent respectively the anti-DNP H.A. test and control groups.

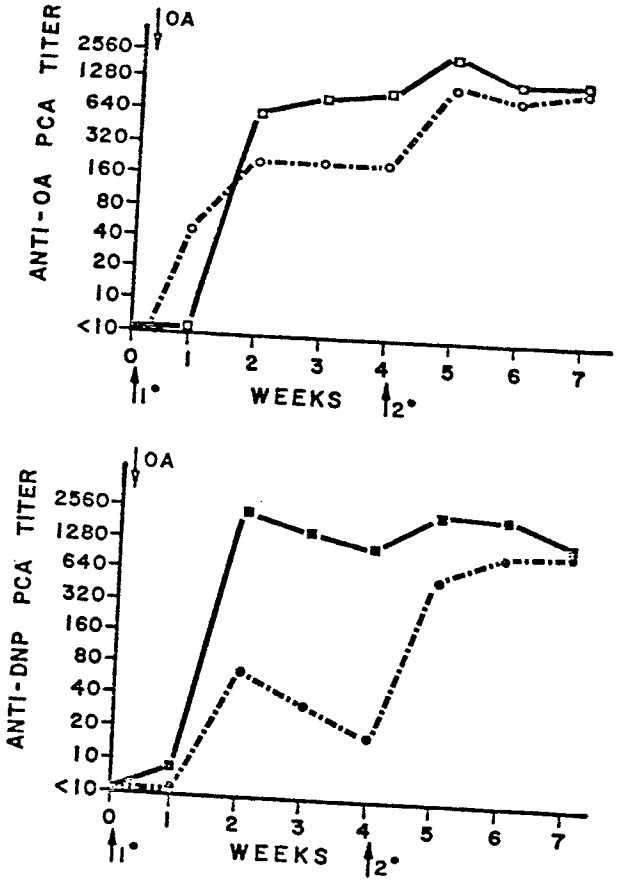
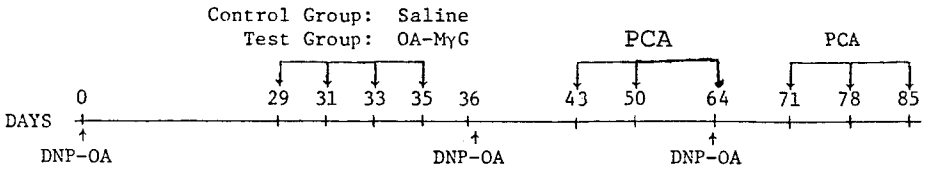


Figure 10: Effect of Native OA on the primary anti-DNP and anti-OA responses.

Two groups of mice were treated with 420 µg of OA i.p. or with saline. Both groups were then immunized with the sensitizing dose of DNP-OA on the same day. The anti-DNP and anti-OA IgE responses of treated (●·· ··● ,○ ·· ··○) and control groups (■—■,□—□) were measured. Both groups of mice were resensitized with the sensitizing dose of DNP-OA on day 29.

TABLE VI

EFFECT OF OA-MγG ON THE ONGOING ANTI-DNP AND ANTI-OA RESPONSES



GROUP	TREATMENT*			PCA TITERS	
	Day	Compound	Day	Anti-DNP	Anti-OA
Control	0	DNP-OA			
	29,31,33,35	Saline			
	36	DNP-OA	43	3160	3250
			50	1100	2700
			64	1100	1300
	64	DNP-OA	71	1400	2500
			78	1250	3600
			85	1000	3100
Test	0	DNP-OA			
	29,31,33,35	OA-MγG			
	36	DNP-OA	43	240	300
			50	160	300
			64	70	300
	64	DNP-OA	71	70	310
			78	275	640
			85	220	220

* All mice received sensitizing doses of DNP-OA on days 0, 36 and 64. Mice in the test group received additional injections of 210 μg of OA-MγG in 0.5 ml of saline on days 29, 31, 33 and 35, whereas the control mice received injections of 0.5 ml of saline only. After the second injection of DNP-OA the mice were bled on days 43, 50 and 64. and again on days 71, 78 and 85 after the third injection of DNP-OA.

of DNP-OA and beginning on day 28, received 4 injections of OA-MγG 210 μg/injection/mouse) at intervals of 2 days, and were resensitized with DNP-OA one day after the last injection of OA-MγG and again on day 64. As is evident from the results listed in Table VII, administration of OA-MγG resulted in a longlasting depression of the capacity of the primed mice to mount anti-DNP and anti-OA IgE responses. Thus, the secondary and tertiary responses of these mice were significantly lower than those of control animals even after the third injection of the sensitizing dose of DNP-OA, which was administered 29 days after the last injection of OA-MγG. As would be expected, by contrast, the control animals mounted typical secondary and tertiary anti-DNP and anti-OA IgE responses.

3) Effect of the dose of OA-MγG on the anti-OA IgE response.

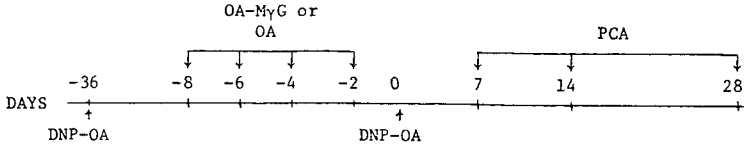
The effect of varying the dose of OA-MγG administered was studied and the results are shown in Table VII. It is evident, that whereas four injections each of 105 or 210 μg of OA-MγG led to marked suppression of secondary anti-OA IgE responses; lower dose of OA-MγG, i.e. 55 μg per injection, did not affect the production of the IgE antibodies. Moreover, in order to establish that effective immunosuppression required the OA moiety to be presented in the form of a conjugate with isologous γ-globulin, the effect of free OA on the secondary IgE response was also investigated. For this purpose, the doses of free OA used corresponded roughly to the amounts of OA present in the conjugates. It is obvious from the results listed in Table VII that administration of free OA over the wide range of 17.5 to 330 μg per injection had no detectable effect on the secondary anti-OA IgE response, and one may conclude that conjugation of OA and MγG was essential for the production of immunosuppressive compounds.

4) Specificity of Immunosuppression with OA-MγG

The specificity of the immunosuppressive effect of OA-MγG

TABLE VII

EFFECT OF THE DOSE OF OA-MγG ON THE ANTI-OA IgE RESPONSE



	COMPOUND*	DAY	ANTI-OA PCA TITERS
	Control	7	3,100
		14	1,200
		28	2,560
TEST 1	OA-MγG		
	210 µg/inj. (85 µg)**	7	300
		14	300
		28	310
	105 µg/inj. (42.5 µg)	7	340
		14	640
		28	310
	55 µg/inj. (21.25)	7	3,000
		14	3,600
		28	2,560
TEST 2	OA		
	330 µg/inj.	7	3,000
		14	3,200
		28	2,560
	170 µg/inj.	7	3,075
		14	2,600
		28	2,560
	70 µg/inj.	7	3,200
		14	2,600
		28	1,500
	35 µg/inj.	7	3,200
		14	2,600
		28	1,280
	17.5 µg/inj.	7	3,200
		14	2,600
		28	1,300

* All mice had received a sensitizing dose of DNP-OA 28 days prior to the beginning of the treatment with OA-MγG or OA, which consisted of 4 injections of the appropriate dose of OA-MγG or OA at 2 day intervals; one day later all mice were given a further sensitizing dose of DNP-OA. The control mice received saline instead of OA-MγG.

** The values in brackets represent the amounts of OA incorporated into the OA-MγG conjugates.

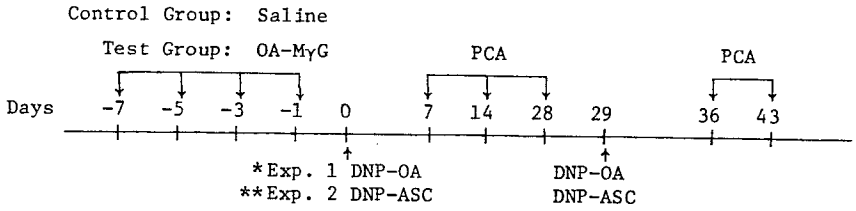
was tested as illustrated by the protocol given in Table VIII. A group of 8 normal mice received four injections of OA-MγG conjugate (210 µg/ mouse) at 2 day intervals; the control group of 8 animals received instead four injections of saline. Both the test and control groups were then subdivided into two groups of 4 mice each and all animals received one day later a sensitizing dose of DNP-OA or of DNP-Asc. After an interval of 29 days, all mice received a secondary sensitization injection of the corresponding antigens as indicated in Table VIII.

As is obvious from the results listed in the lower section of Table VIII, treatment of mice with multiple injections of OA-MγG did not interfere with the subsequent primary and secondary anti-DNP and anti-Asc responses, which were elicited by sensitization with DNP-Asc. On the other hand, both the primary and secondary anti-DNP and anti-OA responses of mice treated with OA-MγG were suppressed when these mice were immunized with the sensitizing dose of DNP-OA.

In another experiment, illustrated in Figure 11, the specificity of the suppressive effect of the OA-MγG conjugate was tested with the aid of two groups of mice which had been primed one month previously with RNase, one of which received the OA-MγG conjugate while the other received saline. Both groups of mice received one day and 28 days later a sensitizing dose of RNase. As is evident from the results in the upper section of Figure 11, injection of OA-MγG had no effect on the secondary and tertiary anti-RNase IgE responses.

TABLE VIII

SPECIFICITY OF IMMUNOSUPPRESSION BY OA-MγG



		Treatment*		PCA Titers			
		Day	Compound	Day	Anti-OA	Anti-DNP	Anti-ASC
EXPERIMENT 1	Control	-7-5-3-1	Saline				
		0	DNP-OA	7	<10	<10	N.T.***
				14	70	160	N.T.
				28	240	220	<10
		29	DNP-OA	36	640	310	<10
				43	300	280	<10
	Test	-7-5-3-1	OA-MγG				
		0	DNP-OA	7	<10	<10	N.T.
				14	<10	<10	N.T.
				28	<10	<10	<10
		29	DNP-OA	36	20	40	<10
				43	20	28	<10
EXPERIMENT 2	Control	-7-5-3-1	Saline				
		0	DNP-ASC	7	<10	<10	<10
				14	<10	310	<10
				28	<10	220	160
		29	DNP-ASC	36	<10	3800	360
				43	<10	1100	100
	Test	-7-5-3-1	OA-MγG				
		0	DNP-ASC	7	<10	<10	<10
				14	<10	270	<10
				28	<10	60	100
		29	DNP-ASC	36	<10	2560	160
				43	<10	1200	80

* In experiment 1, all mice received sensitizing doses of DNP-OA on days 0 and 29. The test mice received additional injections of 210 µg of OA-MγG on days -7, -5, -3 and -1.

** Same protocal as in experiment 1 except the sensitizing dose was DNP-Asc

*** N.T. = not tested.

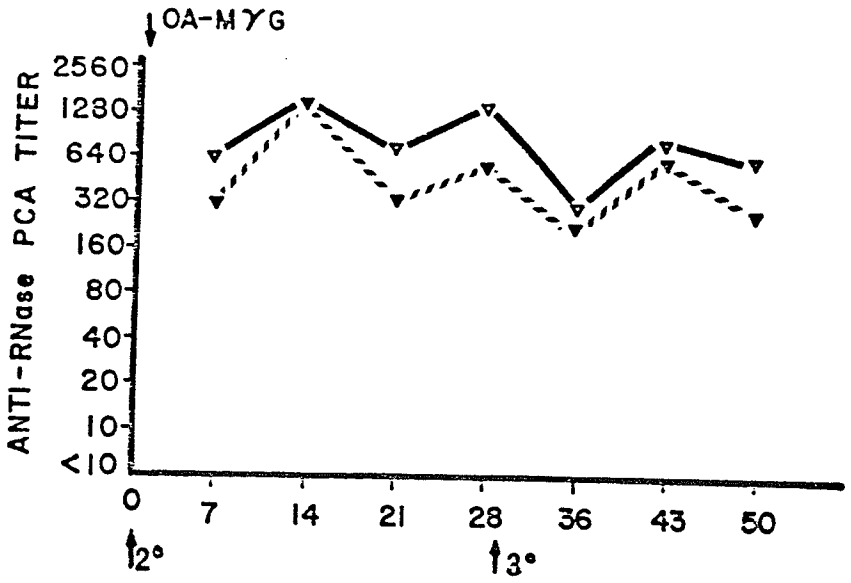


Figure 11: Specificity of treatment with OA-MγG in mice primed with RNase.

Mice were sensitized with RNase three months prior to treatment with OA-MγG. The mice were divided into two groups. One group received an i.p. injection of 500 μg of OA-MγG (▲·····▲) and the other group received a saline treatment (△——△). Both groups were resensitized with RNase in $Al(OH)_3$ on days 0 and day 29. Their anti-RNase responses were measured by PCA in random bred hooded rats.

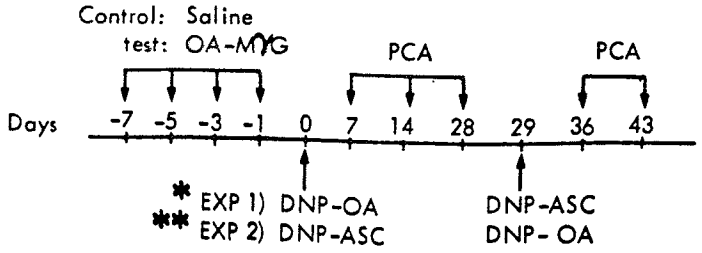
(F) THE CELLULAR MECHANISM OF SUPPRESSION DUE TO THE OA-MγG CONJUGATE

1. Effect of the OA-MγG conjugate on helper cells.

To demonstrate that administration of OA-MγG had a suppressive activity on helper cells the following experiment was performed. The test group of mice received four injections of 210 μg of OA-MγG per mouse at 2 day intervals; the control group received four injections of saline. Each group of mice was then subdivided into two sub-groups and each of the sub-groups was immunized with the sensitizing dose of DNP-OA, or with DNP-Asc. Furthermore, as illustrated in Table IX, each of the four sub-groups of mice received a sensitizing dose of the reciprocal antigen on day 29. From the results listed in this Table it is evident that pretreatment of mice with OA-MγG suppressed the IgE responses to DNP-OA but did not affect the IgE responses to DNP-Asc, irrespective of the order of sensitization with these two antigens. Thus, sensitization with DNP-OA of mice pretreated with the OA-MγG conjugate (Table IX Exp.1) resulted in suppressed primary anti-DNP and anti-OA IgE responses whereas sensitization with DNP-Asc of OA-MγG pretreated mice resulted in both anti-DNP and anti-Asc responses which were similar to those of control mice which had received saline instead of OA-MγG (Table IX, Exp. 2). Moreover, as is evident from the results listed in Table IX, Exp. 2, the tolerogenic effect of OA-MγG in normal mice was long lasting leading to depressed IgE responses of both DNP and OA when the primary and secondary sensitizing antigens was DNP-Asc and DNP-OA respectively. In contrast, the secondary anti-DNP and anti-Asc IgE antibody responses of OA-MγG treated mice, induced by a sensitizing dose of DNP-Asc was not affected by a primary sensitizing dose of DNP-OA (Table IX, Exp. 1).

TABLE IX

EFFECT OF OA-MγG ON HELPER "T" CELL ACTIVITY



	Group	Treatment		PCA Titers			
		Day	Compound	Day	Anti-DNP	Anti-OA	Anti-ASC
EXP 1	Control	-7-5-3-1	Saline				
		0	DNP-OA				
				7	<10	<10	NT ***
				14	160	70	NT
				28	220	240	<10
		29	DNP-ASC				
				36	425	300	<10
				43	1200	950	320
	Test	-7-5-3-1	OA-MγG				
		0	DNP-OA				
				7	<10	<10	<10
				14	<10	<10	<10
				28	<10	<10	160
		29	DNP-ASC				
				36	20	<10	<10
				43	1200	<10	260
EXP 2	Control	-7-5-3-1	Saline				
		0	DNP-ASC				
				7	<10	<10	<10
				14	310	<10	<10
				28	220	<10	160
		29	DNP-OA				
				36	640	<10	<10
				43	1100	280	<10
	Test	-7-5-3-1	OA-MγG				
		0	DNP-ASC				
				7	<10	<10	<10
				14	270	<10	<10
				28	60	<10	100
		29	DNP-OA				
				36	160	28	65
				43	80	20	20

* Legend, next page

TABLE IX

EFFECT OF OA-MγG ON HELPER "T" CELL ACTIVITY

* Legend

* In experiment 1, all mice received a primary sensitization with DNP-OA on day 0 and the second sensitization with DNP-Asc on day 29. The test mice received additional injections of 210 μg of OA-MγG on days -7, -5, -3, and -1

** In experiment 2, all mice received a primary sensitizing dose of DNP-Asc on day 0, a sensitizing dose of DNP-OA on day 29. All test mice received additional injections of 210 μg of OA-MγG on days -7, -5, -3 and -1.

*** N.T. = not tested.

The recovery of the anti-DNP IgE response in the OA-MγG pretreated mice after successive sensitization with DNP-OA and DNP-Asc clearly demonstrates that the DNP specific B_ε cells were not affected by the OA-MγG conjugate and that the observed suppression was probably due to a suppressor cell acting on the helper T cell or to the tolerization of the latter cells.

2. Attempt to break tolerance by transfer of normal or DNP-OA primed cells into mice tolerized with OA-MγG

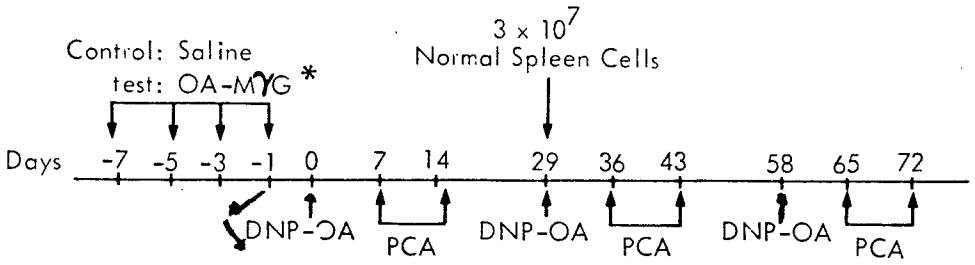
As stated above, the results listed in Table IX were interpreted as indicating that helper T cells were the main cells affected by the treatment of mice with OA-MγG. In an attempt to determine if the observed suppression involved inactivation of these cells, or was due to an active suppressive phenomenon caused by suppressor cells the experiments outlined in the protocol given in Tables X and XI were performed.

First as shown in Table X, two groups of normal mice received at 2 day intervals, four i.p. injections of 210 μg of OA-MγG each and two other groups were treated with saline. All four groups were sensitized one day after the last injection with the sensitizing dose of DNP-OA and their anti-DNP and anti-OA IgE antibodies were determined 7 and 14 days later. On day 29 after primary immunization, normal spleen cells (3×10^7/mouse) were injected i.p. into one group which had been initially treated with OA-MγG and into another group which had been initially treated with saline; the remaining two groups did not receive any cells and served as controls for these two groups, respectively.

All four groups were then given an i.p. booster injection of the sensitizing dose of DNP-OA within four hours after the cell transfer

TABLE X

EFFECT OF NORMAL SPLEEN CELLS ON IgE RESPONSES OF TOLERIZED MICE

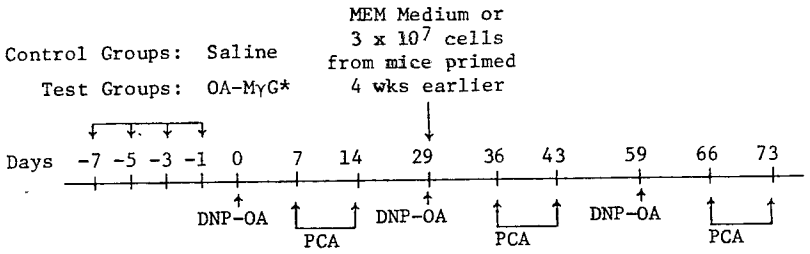


		PCA Titers			
	Day	Mice Receiving No Cells on Day 29		Mice Receiving Cells on Day 29	
		Anti-DNP	Anti-OA	Anti-DNP	Anti-OA
Control	7	10	10	10	10
	14	1150	640	1150	640
	36	300	1125	300	2900
	43	640	690	640	1000
	65	1000	1280	1000	1280
	72	870	640	820	640
Test	7	10	10	10	10
	14	10	10	10	10
	36	25	80	23	90
	43	90	80	20	10
	65	220	90	85	30
	72	80	80	80	10

* The test mice were given four i.p. injections of 210 μg of OA-MγG on days -7,-5,13 and -1 and an i.p. sensitizing dose of DNP-OA on days 0. One subgroup of mice as well as one subgroup of the control mice were administered 3 x 10^7 spleen cells from normal mice. The other two corresponding subgroups received MEM on day 29. All mice received two additional sensitizing doses of DNP-OA on days 29 and 58.

TABLE XI

EFFECT OF DNP-OA PRIMED SPLEEN CELLS ON IgE RESPONSE OF TOLERIZED MICE



		PCA Titers			
	Day	Mice Receiving No Cells on Day 29		Mice Receiving Cells on Day 29	
		Anti-DNP	Anti-OA	Anti-DNP	Anti-OA
Control	7	<10	<10	<10	<10
	14	1140	640	1140	640
	36	390	640	1025	1280
	43	640	890	1125	1250
	66	320	900	700	1200
	73	240	640	640	1000
Test	7	<10	<10	<10	<10
	14	<10	<10	<10	<10
	36	100	320	65	85
	43	80	280	25	80
	66	180	85	30	80
	73	80	280	20	80

* The test mice were given four i.p. injections of 210 µg of OA-MγG on days -7, -5, -3 and -1 and an i.p. sensitizing dose of DNP-OA on day 0. One subgroup of mice as well as one subgroup of the control mice were administered 3 x 10^7 spleen cells from mice which had been sensitized to DNP-OA 4 weeks before sacrifice. The other two corresponding subgroups received MEM medium on day 29. All mice received two additional sensitizing doses of DNP-OA on day 29 and 59.

and a further injection 29 days later. As is evident from the results listed in Table X, the anti-DNP and anti-OA IgE responses of the test group of mice which had received OA-MγG and no spleen cells remained profoundly suppressed for a period of at least 72 days in spite of the fact that the mice had received three sensitizing injections of DNP-OA at intervals of 29 days. Moreover, the transfer of normal spleen cells into mice, which had been immunosuppressed with OA-MγG, was ineffective in counteracting the tolerogenic effect of OA-MγG (which had been administered 29 days earlier) in spite of the fact that these mice received two additional sensitizing doses of DNP-OA on days 30 and 59, after the last injection of OA-MγG. It is also obvious from the results given in Table X, that transfer of normal spleen cells into mice which had been immunized with DNP-OA 29 days prior to cell transfer, did not depress their capacity to mount IgE antibody responses to either DNP or OA.

Using the similar experimental protocol illustrated in Table XI the effect of the transfer of 3×10^7 spleen cells from sensitized animals into immunosuppressed mice was investigated on the IgE responses of the latter. The results listed in Table XI demonstrated not only that the immunosuppression with OA-MγG was not reversed or counteracted by the transfer of primed cells, but more importantly, that the immunosuppressed hosts had acquired the capacity to depress even the responsiveness of primed cells. Hence, it may be inferred that administration of OA-MγG triggered an "active" immunosuppressive process which involves probably the generation of carrier-specific suppressor cells.

In the experiments illustrated in Tables X and XI normal mice had been first immunosuppressed with OA-MγG and received on day 29 spleen

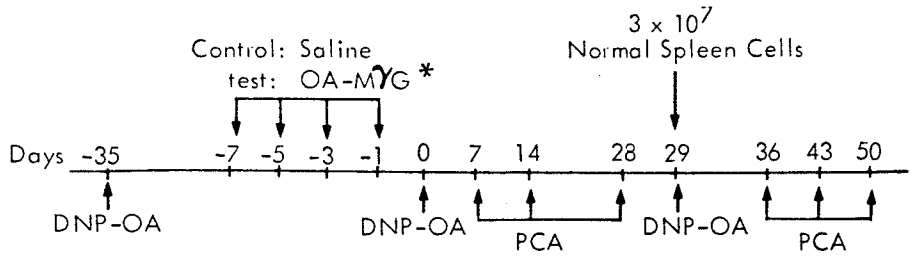
cells from normal or DNP-OA primed mice in an attempt to reverse their tolerant state. By contrast, in the experiments about to be described (Table XII, XIII) mice were first primed with a sensitizing dose of DNP-OA and given 29 days later four injections of OA-MγG (210 μg/mouse) at two day intervals; the group of control mice received saline instead of the OA-MγG conjugate. One day after the last injection of OA-MγG, the test and control mice were boosted with a sensitizing dose of DNP-OA and both groups received, 29 days later, 3×10^7 spleen cells from normal mice or from mice which had been primed 28 days earlier with a sensitizing dose of DNP-OA. Within 4 hours after cell transfer, the mice received an additional injection of the sensitizing dose of DNP-OA and their IgE antibody responses were monitored at weekly intervals. Additional control groups of animals received no cells. As is evident from the results in Tables XII and XIII, transfer of cells from normal or primed mice into the control group of mice did not affect the anti-DNP or anti-OA IgE antibody responses of these animals. By contrast the IgE responses of mice which had been sensitized with DNP-OA and then immunosuppressed with OA-MγG remained depressed in spite of receiving cells from normal or primed mice as well as two additional booster injections of the sensitizing dose of DNP-OA. These results demonstrate, therefore, that immunosuppression can be induced readily also in presensitized animals and that the state of unresponsiveness cannot be reversed by the transfer of normal or even primed cells. Hence, these data reinforce the inference that injection of OA-MγG results in active suppression probably by the generation of suppressor cells.

3. Transfer of OA-MγG induced tolerance into normal mice.

In an attempt to substantiate that the observed immuno-

TABLE XII

EFFECT OF NORMAL SPLEEN CELLS ON DNP-OA PRIMED AND TOLERIZED MICE



	Day	PCA Titers			
		Mice Receiving No Cells		Mice Receiving Normal Cells on Day 29	
		Anti-DNP	Anti-OA	Anti-DNP	Anti-OA
Control	7	3160	3250	3160	3250
	14	1100	2700	1100	2700
	28	2560	1300	2560	1300
	36	1300	3400	1300	3400
	43	1200	2560	1100	2900
	50	1800	3150	1000	3100
Test	7	240	300	240	300
	14	160	300	160	300
	28	70	310	70	310
	36	275	640	300	640
	43	160	200	200	200
	50	180	280	220	220

* legend, next page

TABLE XII

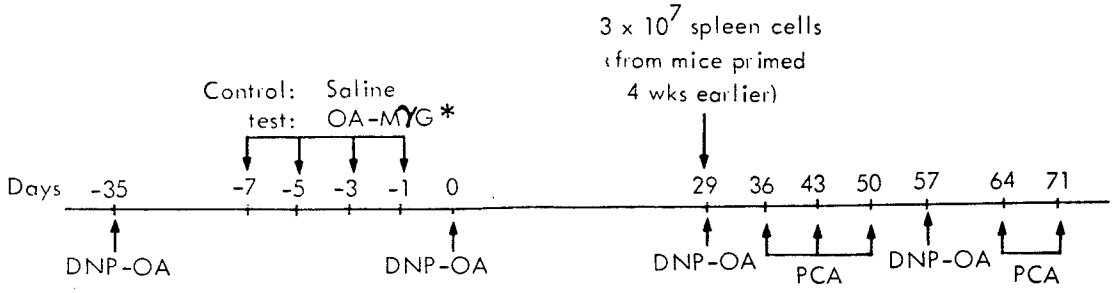
EFFECT OF NORMAL SPLEEN CELLS ON DNP-OA PRIMED AND TOLERIZED MICE

* Legend

All mice were presensitized with DNP-OA on day - 35. The test mice were given four i.p. injections of 210 μg of OA-MγG on days -7, -5, -3 and -1 and resensitized with DNP-OA on day 0. One subgroup of these mice as well as one subgroup of the control mice were administered 3×10^7 spleen cells from normal mice. The other two corresponding subgroups received MEM on day 29. All mice received an additional sensitizing dose of DNP-OA on day 29.

TABLE XIII

EFFECT OF DNP-OA PRIMED SPLEEN CELLS ON DNP-OA PRIMED AND TOLERIZED MICE



		PCA Titers			
	Day	Mice Receiving No Cells		Mice Receiving Cells on Day 29	
		Anti-DNP	Anti-OA	Anti-DNP	Anti-OA
Control	36	1050	2560	1150	2800
	43	1400	2560	1400	2560
	50	280	640	1000	700
	64	1050	1200	900	640
	71	280	1125	260	1100
Test	36	280	640	640	640
	43	100	240	330	300
	50	80	160	290	160
	64	75	65	320	220
	71	40	70	80	280

* Legend next page

TABLE XIII

EFFECT of DNP-OA PRIMED SPLEEN CELLS ON DNP-OA PRIMED AND TOLERIZED MICE

*Legend

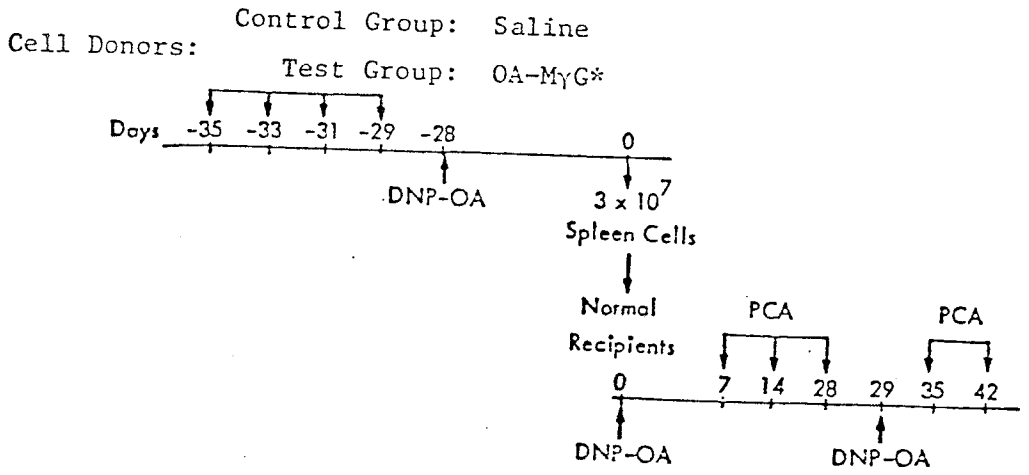
All mice were presensitized with DNP-OA on day -35. The test mice were given four i.p. injections of 210 μg of OA-MγG on days -7, -5, -3 and -1 and a sensitizing dose of DNP-OA on day 0. One subgroup of these mice as well as one subgroup of the control mice were administered 3×10^7 spleen cells from mice which had been sensitized to DNP-OA four weeks before sacrifice. The other two corresponding subgroups received MEM on day 29. All mice received two additional sensitizing doses of DNP-OA on days 29 and 59.

suppression induced by OA-MγG was due to suppressor cells specific for OA, normal mice received four injections of OA-MγG (210 μg/mouse) at two day intervals and one day later, a sensitizing dose of DNP-OA; the control group received saline instead of OA-MγG. Four weeks later these two groups of mice were sacrificed and suspensions of 3×10^7 spleen cells of each of the two groups were injected i.p. into normal mice. The two groups of recipient mice, as well as another control group which had not received any cells, were then administered two sensitizing doses of DNP-OA, one within four hours after cell transfer and the other 29 days later. It is evident from the results of this experiment, listed in Table XIV, that mice which had received spleen cells from OA-MγG treated donors demonstrated reduced capacity of mounting IgE responses to the sensitizing dose of DNP-OA, whereas, mice which had received spleen cells from primed mice or no cells at all responded to the sensitizing dose of DNP-OA as manifested by their ability to produce IgE antibodies to OA and DNP.

In a similar experiment, (Table XV) one of two groups of DNP-OA primed mice (day-42) was tolerized with four repeated injections of OA-MγG and the other group was treated with saline. Both groups were then given a sensitizing dose of DNP-OA one day later. After 7 days the mice were sacrificed and 3×10^7 spleen cells from the tolerized and control groups were injected i.p. into normal recipients which were then administered a sensitizing dose of DNP-OA. From the results, summarized in Table XV, it is evident that suppressor cells were present in spleens of mice eight days after the last injection of OA-MγG since the anti-OA and anti-DNP IgE responses of the cell

TABLE XIV

TRANSFER OF TOLERANCE INTO NORMAL MICE WITH SPLEEN CELLS OF OA-MγG TREATED AND DNP-OA PRIMED MICE

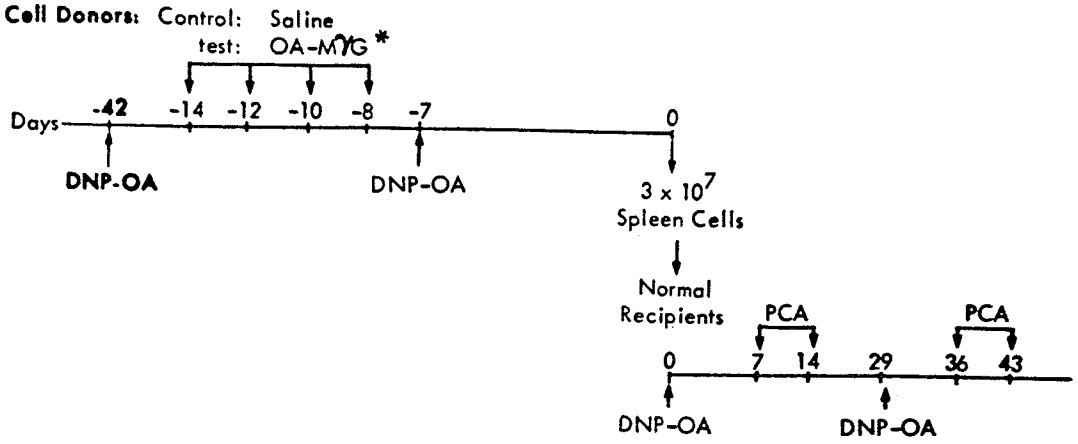


Day	PCA Titers					
	Control Group: Received No Cells		Test Groups			
			Recipients of Primed Spleen Cells		Recipients of Tolerized Spleen Cells	
	Anti-DNP	Anti-OA	Anti-DNP	Anti-OA	Anti-DNP	Anti-OA
7	80	<10	65	85	20	80
14	850	210	160	80	75	80
28	250	640	210	640	25	80
35	700	1300	1100	1280	280	160
42	1280	1400	1280	1280	80	280

* Donors of tolerized cells received i. p. injections of 210 µg each of OA-MγG on days -35, -33, -31 and -29, followed by a sensitizing dose of DNP-OA on day -28. The spleen cells (3×10^7) of these mice were transferred into normal syngeneic mice four weeks later, i.e. on day 0. Donors of primed cells received only a sensitizing dose of DNP-OA 28 days before sacrifice. The cell recipients were sensitized with DNP-OA on days 0 and 29.

TABLE XV

TRANSFER OF TOLERANCE INTO NORMAL MICE WITH DAY 7 SPLEEN CELLS OF DNP-OA PRIMED AND TOLERIZED MICE



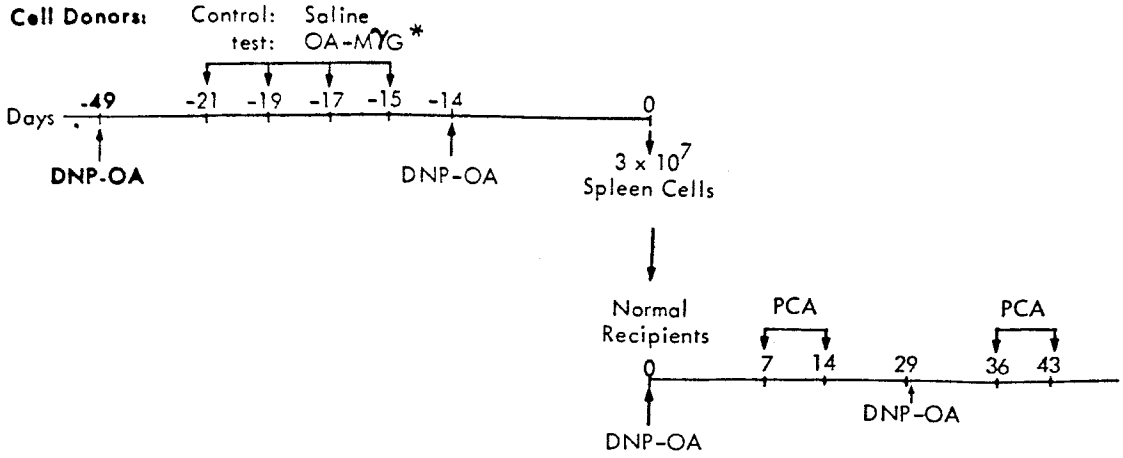
Day	PCA Titers of Recipient Mice Which had Received					
	No Cells		Primed Cells		Tolerized Cells	
	Anti-DNP	Anti-OA	Anti-DNP	Anti-OA	Anti-DNP	Anti-OA
7	70	10	240	245	245	160
14	5120	4100	1024	900	280	20
36	3100	4200	3100	4200	1050	640
43	4200	5100	4600	4900	1200	1100

* Donors presensitized with DNP-OA on day -42, received i.p. injections of 210 µg each of OA-MγG on days -14, -12,-10 and -8, followed by a sensitizing dose of DNP-OA on day -7. The spleen cells (3×10^7) of these mice were transferred into normal syngeneic mice on day 0. Donors of primed cells received sensitizing doses of DNP-OA -42, and -7 days before sacrifice. The recipients were sensitized with DNP-OA on days 0 and 29.

recipients were suppressed. However, the anti-DNP and anti-OA responses of recipients of day 14 spleen cells of OA-MγG donors were not suppressed indicating that these suppressor cells are short lived (Table XVI).

TABLE XVI

FAILURE TO TRANSFER TOLERANCE INTO NORMAL MICE WITH DAY 14 SPLEEN CELLS OF DNP-OA PRIMED AND TOLERIZED MICE



Day	PCA Titers of Mice Which Had Received:					
	No Cells		Primed Cells		Tolerized Cells	
	Anti-DNP	Anti-OA	Anti-DNP	Anti-OA	Anti-DNP	Anti-OA
7	10	10	80	1024	80	1024
14	5100	3100	900	900	1000	1100
36	3600	4200	3600	4400	3600	4200
43	2560	5100	3600	4900	3600	5100

* Donors, presensitized with DNP-OA on day -49, received i.p. injections of 210 µg each of OA-MγG on days -21, -19, -17, and -15 followed by a sensitizing dose of DNP-OA on day -14. The spleen cells (3×10^7) of these mice were transferred into normal syngeneic mice on day 0. Donors of primed cells received a sensitizing doses of DNP-OA -49 and -14 days before sacrifice. The recipients were sensitized with DNP-OA on days 0 and 29.

DISCUSSION

The findings in this study to date support the conclusion that OA-MγG conjugates with an average molecular composition corresponding to $(OA)_{12}$- $(M\gamma G)_2$ were capable of suppressing specifically both the primary and secondary anti-DNP and anti-OA IgE responses which were induced by immunization of mice with a sensitizing dose of DNP-OA. The OA-MγG conjugates were prepared by coupling OA to MγG with the use of glutaraldehyde. The molecular weight and sedimentation coefficient of the conjugate confirmed the results obtained by the radiolabelling of MγG with ^{125}I and then forming the conjugate with OA. Employing the specific activity and optical characteristics of the conjugate, the empirical formula of OA-MγG was calculated.

The presence of high molecular weight OA-OA hybrids was not detected either by immunodiffusion or immunoelectrophoresis (Figures 2,3). No spurs were seen when OA-MγG was tested against anti-OA and anti-NMS in Ouchterlony analysis. The bands of identity clearly demonstrated the absence of polymerized OA in the fraction containing OA-MγG (Figure 2).

The administration of OA-MγG into mice resulted in long term suppression of their ability to mount primary or secondary anti-OA or anti-DNP IgE responses on immunization with DNP-OA. The injection of unmodified OA polymerized OA or OA-RγG conjugates, induced only a transient diminution of the primary anti-OA and anti-DNP IgE responses (Tables II, III, IV,V). Moreover, further immunization of these animals with a sensitizing dose of DNP-OA resulted in strong IgE responses approaching or exceeding the levels of antibodies produced by control animals. In addition, the results listed in Table VI, clearly demonstrated that the

suppressive effect of OA-MγG was dose dependent. By contrast, equivalent doses of OA did not affect significantly the anti-OA IgE response. Thus, it was concluded that the production of suppressive conjugates required the use of isologous gamma globulin as carrier. Furthermore, the injection of OA-MγG or OA into normal mice, 14 days prior to sensitizing dose of DNP-OA on day 0, led to a marked diminution of the anti-OA and anti-DNP reaginic responses of mice treated with OA-MγG whereas an anamnestic response (anti-OA and anti-DNP) was clearly induced in mice administered OA (Table V). Thus, these results further support the previous conclusion that OA must be presented in the form of a conjugate with isologous gamma globulin in order to act as a suppressive agent.

The specificity of the observed immunosuppression was demonstrated by the fact that the IgE response to an unrelated antigen, such as RNase, was not affected by the administration of the tolerogenic conjugate, OA-MγG, (Figure 11). Furthermore, administration of OA-MγG conjugates did not affect the formation of anti-DNP IgE antibodies in response to immunization of mice with DNP-Asc (Table VII). Hence one may infer that the observed immunosuppression involved tolerization of the animals with respect to the carrier, OA, probably by the generation of carrier-specific suppressor cells which have a negative effect on helper T cells. This inference is in agreement with the results of many studies which demonstrated that tolerization with respect to the determinations of the carrier of a given antigen interferes with the cooperation of the appropriate helper T cells and percursor B cells involved in the immune response to the antigen in question (157).

This interpretation of the results was confirmed in the experiment described in Table IX, where the effect of OA-MγG conjugates on helper T cells was examined. Mice were treated with OA-MγG conjugates, followed

by a primary immunization on day 0 with DNP-OA or DNP-Asc, and by a second sensitization of the reciprocal antigen on day 29. The recovery of the anti-DNP response in the OA-MγG pretreated mice after successive sensitization with DNP-OA and DNP-Asc, clearly demonstrated that the DNP specific B_ε cells were not affected by the OA-MγG conjugates and the observed immunosuppression was probably due either to a suppressor cell acting on the helper T cells or that the helper T cells were "turned off" by the conjugate.

The slow rate of clearance of ^{125}I-labelled OA-MγG by comparison with that of ^{125}I-labelled OA, may be considered as indirect evidence for the conjugate being markedly less immunogenic by comparison with the immunogenicity of OA. This inference was based on an increasing body of evidence favouring the hypothesis that potential helper T cells possess two recognition units, one for the antigen and the other for the products of the MHC gene complex and that activation of helper T cells to most soluble antigens occurs only if the antigen is processed by macrophages and presented to the T cell membrane in association with the appropriate gene product encoded in the I region of the MHC complex (41, 157,158). By contrast, the processing of antigens by macrophages does not appear to be necessary for the activation of suppressor T cells; in fact, the very opposite appears to be the case (131,159). Hence, one may visualize that interaction of only one receptor on T cells with a conventional antigen in the absence of a concerted interaction of the other receptor with autologous MHC antigens, would result in activation of suppressor T cells; under these circumstances, the antigen would remain essentially nonimmunogenic. On the basis of this interpretation of the mechanism

controlling the activation of helper and suppressor T cells, the finding that the clearance of OA-MγG conjugate was very much slower than that of the unmodified OA is in accord with the fact that only one injection of OA-MγG was sufficient to suppress the IgE responses for a prolonged period and may be plausibly attributed to the possibility that these conjugates would not be readily processed by macrophages or would stimulate macrophages without Ia antigens (160). Consequently, these conjugates would not become associated with Ia-antigens of the H-2 complex and would, therefore, stimulate the generation of suppressor T cells rather than helper T cells.

In an attempt to elucidate the cellular mechanism underlying the observed immunosuppression by the tolerogenic conjugate, adoptive transfer experiments were designed using normal, sensitized or tolerized non-irradiated recipients and spleen cells from tolerized, sensitized or normal donors in different combinations. It is evident from the data listed in Table XIV that the transfer of cells from donors which had been tolerized with OA-MγG had a profoundly suppressing effect on the capability of non-immune syngeneic recipients to mount IgE responses to either OA or DNP. Hence, it may be inferred from these data that the tolerogen leads to the generation of suppressor cells over an extended period of time. Similarly, the attempt to break tolerance, which had been induced by pretreatment of the recipients with OA-MγG by the transfer of cells from either normal or DNP-OA primed mice was unsuccessful (Tables X,XI,XII,XIII). From these results one may infer that the balance between suppressor cells and helper T cells in these recipients was tilted in favour of suppressor cells.

The generation of suppressor cells over an extended period of time may indicate also that OA-MγG conjugates were lodged in an organ,

such as Peyer's patches, and induced the formation of suppressor cells. In such organs, the conjugates would not be degraded or eliminated by the host recticulo-endothelial system, but may specifically activate cells which dampen the reaginic response. Ts cells have been shown to be induced in Peyer's patches and these cells migrated out to the other organs such as the spleen (138,139). The possible carry over of the suppressive conjugate in the cell transfer experiments is unlikely since the conjugate, with a half-life of approximately three days and with the transfer of cells 29 days after treatment, approximately 1.6 x 10^{-3} mg of the conjugate would be left in the donors which is far below the concentration of OA-MγG needed to induce suppression of normal mice (Table XV). Furthermore, 1.1 μg of the conjugate was left in the spleen of mice treated with radioactive OA-MγG-I^{125}, 14 days after the injection of the conjugate, again far below the threshold values necessary for the induction of tolerance.

It must be stressed that the hemagglutinating antibody response was not affected by the OA-MγG treatments and if the observed suppression was due to neutralization of the specific antibody, then the hemagglutinating antibody response should have been neutralized also. Unless IgE antibody has a greater affinity for the antigen than its hemagglutinating counterpart - there is no prior reason to believe that it has - the OA-MγG should be processed by the cells that affect the IgE and hemagglutinating antibody responses in identical fashion.

From the results of all these experiments, it may be concluded that administration of OA-MγG generated suppressor cells which had the ability of depressing the potential of both normal and primed animals to mount reaginic antibody responses to ovalbumin and to a hapten attached

to it. This evidence also strongly suggests that helper T cells are probably the target cells in the overall mechanism responsible for suppression. In principle, it may be visualized that suppressor cells may act either on T helper cells or on the appropriate B cells or on both. It ought to be pointed out however, that the mechanism of suppression induced by conjugates of isologous γ-globulins with antigens appears to be different from that induced by conjugates of the same globulins with simple haptens, since in the latter case no suppressor cells were detected in adoptive transfer experiments utilizing different combinations of cells in intact and x-irradiated recipients (6).

Other cellular mechanism may also explain the immunosuppression achieved in this study. Basten et al (161) suggested that there may be a population of precursor suppressor T (Tsp) cells which possess Fc receptors specific for mouse immunoglobulins and that these cells, on interaction with antigen-antibody complexes, were differentiated into effector suppressor T (Tse) cells specific for the antigen. Hence, it is conceivable that the multimolecular conjugates of OA_{12}-$M\gamma G_2$ prepared in this study were being recognized as antigen-antibody complexes and were thus capable of activating the Fc^+ Tsp cells. However, it is to be stressed that, although the generation of suppressor cells by the administration of these conjugates has been unequivocally demonstrated in this study, the nature of these cells remains to be elucidated. Provisionally, the finding that injection of OA-MγG induced suppressor cells which had the ability of depressing the potential of both normal and primed animals to mount reaginic antibody responses not only to ovalbumin, but also to the hapten attached to it, may be interpreted as indicating, that these cells belonged at least in part to a Tse cell population interacting with the carrier protein which had been modified by attachment to MγG.

CHAPTER 3

SPECIFIC SUPPRESSION OF REAGINIC AND HEMAGGLUTINATING ANTIBODY TO DOG ALBUMIN (DA) BY ADMINISTRATION OF SOLUBLE DA.

INTRODUCTION

Reaginic antibody responses have been suppressed specifically by treatment of mice with modified antigens (reviewed in 6). However, the administration of soluble antigen into normal mice or mice primed with antigen affected only transiently the IgE response of the recipients. Thus, while Takatsu and Ishizaka (132) have suppressed the reaginic antibody response to OA with the soluble form of the antigen, Katz and collegues (10,11) on the other hand, demonstrated only marginal inhibitory effects using soluble OA. The suppression with native antigen was short lived and the response was boosted easily. Similarly, Lee and Sehon (6) Usui and Matuhasi (12) and Filion (2nd chapter of thesis) reported, at best, a transient suppression of the reaginic antibody response in mice treated with soluble OA. However, Dessein et al (76) in their study of the IgE response to the synthetic antigen GAT, revealed both antigen specific H-2 linked Ir gene control and immunoglobulin class specific regulation of the IgE response. Moreover, Drs. Wie in this laboratory observed that soluble DA, as well as conjugates of DA-PEG, suppressed the anti-DA IgE response. Thus, a study was undertaken to establish the optimal conditions for the induction of anti-DA IgE response and to determine if the primary and secondary anti-DA IgE responses could be abrogated specifically by soluble DA. The cellular mechanism(s) involved in the induction and maintenance of the unresponsive state is described in the next chapter.

MATERIALS AND METHODS

ANIMALS: Inbred 6- to 12-week old (C57BL/6 x DBA/2)F_1 male mice (designated as $B_6D_2F_1$) were purchased from either Timco Ltd., Houston, Texas, or Gunton Vivarium, Gunton, Manitoba and random-bred hooded rats obtained from Bio-Breeding Laboratories Ltd., Ottawa, Ontario.

PROTEIN ANTIGENS AND OTHER REAGENTS: All protein antigens and other reagents were obtained from commercial sources, i.e. dog albumin (DA), ovalbumin (OA) and α-amylase (α-A) from Sigma Chemical Co., St. Louis, Mo.; bovine gamma globulin (BγG) from Nutritional Biochemicals, Cleveland, Ohio; 2-mercaptoethanol from BDH Biochemicals Ltd., Poole, England; ^{125}I in the form of $Na^{125}I$ from Amersham/Searle Corporation, Arlington Heights, Illinois ; Eagles minimum essential medium (MEM) and fetal calf serum (FCS) from Gibco, San Diego, Ca.; Hepes (N-2-hydroxyethylpiperazine-N'-2 ethane-sulfonic acid) from Calbiochem, San Diego, Ca.; agarose from Olac 1976 Ltd., Bicestor Oxon, England and Miles Lab., Elkhart, In.; 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl from Story Chemical Corp., Muskegen, Mich. The dinitrophenylated proteins, namely DNP_3-OA, and DNP-NMS (NMS = normal mouse serum) were prepared as previously described in Chapter 2. The developing antibody (guinea pig anti-mouse IgG) for detection of IgG plaques was kindly donated by Dr. B.G. Carter.

HALF-LIFE STUDIES OF ^{125}I-DA: DA (400 ug) was radiolabelled with ^{125}I using the chloramine T method as described in Chapter 2, p. 43 . Radio-active ^{125}I-DA, with a specific activity as indicated in the experiments, was injected into mice primed with DA on day -28. The rate of disappearance of ^{125}I from the body and the circulation was determined by the measurement of residual gamma radiation in the whole body or in 25 µl of blood at different times, in a Beckman Gamma 300 Counter (Beckman

Instruments Inc., Fullerton, Ca.).

IMMUNIZATION AND MEASUREMENTS OF IMMUNE RESPONSES: Optimal murine IgE responses were induced by i.p. injection of a sensitizing dose of antigen (i,e, 1 μg of DA or OA and 10 μg of α-A) suspended with 1 mg of freshly prepared $Al(OH)_3$ in 0.5 ml of saline. The magnitude of the reaginic antibody response was measured by the passive cutaneous anaphylaxis (PCA) assay in rats, as previously described in Chapter 2, p. 39 . The presence of DA-specific antibodies other than reagins in sera of immunized mice was demonstrated by the passive HA procedure (described in Chapter 2, p.40) utilizing conjugates of sheep red blood cells (SRBC) with DA sensitized by crosslinking with glutaraldehyde (150). The nature of the agglutinating antibodies i.e., if they were primarily IgM, or IgM plus IgG antibodies, was established in terms of their sensitivity to reduction by treatment with 0.1 M 2-mercaptoethanol (2-ME) (3). For suppression of the IgE response, soluble DA was administered i.p. in different doses as indicated in the appropriate experiments.

ASSAY FOR PLAQUE FORMING CELLS: For the detection of direct and indirect anti-DA PFC, a modified Jerne hemolytic plaque technique (162) was employed. The antigen coated target cells (DA-SRBC) were prepared by the addition of 50 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl in 1 ml of saline to a mixture of 2 ml of packed SRBC (washed 5 times in saline) and 48 mg of DA in 6 ml of saline. The mixture was incubated for 10 min at 37°C and washed three times in phosphate buffered saline containing 5% FCS (FCS-PBS). All PFC responses are reported as arithmetic mean of 2 determinations.

RESULTS

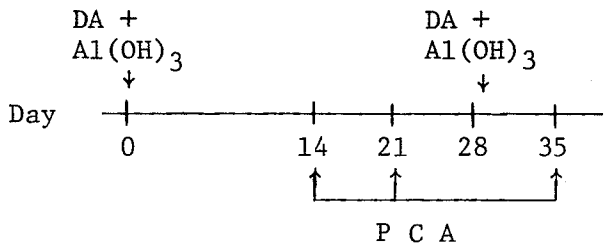
A) INDUCTION AND KINETICS OF ANTI-DA IgE ANTIBODY RESPONSES: The induction of anti-DA IgE antibody responses was performed in $B_6D_2F_1$ mice by the i.p. injection of varying doses (i.e., 0.1, 1, 10, or 100 µg) of DA in the presence of 1 mg of $Al(OH)_3$ on days 0 and 28. As shown in Table XVII, all doses of antigen failed to induce a prominent and prolonged primary anti-DA IgE response. On the other hand, a strong secondary anti-DA IgE response was produced in mice receiving a second dose of 1 or 10 µg of DA in $Al(OH)_3$, whereas, 0.1 and 100 µg of DA were ineffective in this respect. Hence, 1 µg of DA incorporated in $Al(OH)_3$ was chosen as the sensitizing dose of antigen for all future experiments.

The conditions leading to optimal secondary anti-DA response were defined by varying the time interval between primary and secondary sensitization. Thus, three groups of mice immunized with a sensitizing dose of DA in $Al(OH)_3$ on day -21, -14 or -7, were resensitized on day 0 (Table XVIII). Secondary anti-DA IgE and H.A. responses (i.e. high and sustained levels of antibody) were produced by mice, immunized on days -21 and 0 whereas the other groups of mice (i.e. challenged either on day -14 or -7 and on day 0) had lower levels of IgE and H.A. antibodies. However, when the period of time between injections of DA in $Al(OH)_3$ was increased to 28 or 35 days, the IgE or H.A. secondary antibody titers of these mice were comparable to those obtained with a time interval of 21 days (data not shown).

The kinetics of the anti-DA IgE and H.A. responses were described also. As reported in Figure 12, a single injection of DA in $Al(OH)_3$ induced essentially no primary IgE response and only a moderate H.A. antibody response. Resensitization of these mice with DA in $Al(OH)_3$ on days 29 and 58 resulted in the production of secondary and tertiary IgE

TABLE XVII

EFFECT OF ANTIGEN DOSE ON THE PRODUCTION IgE RESPONSE IN $B_6D_2F_1$ MICE

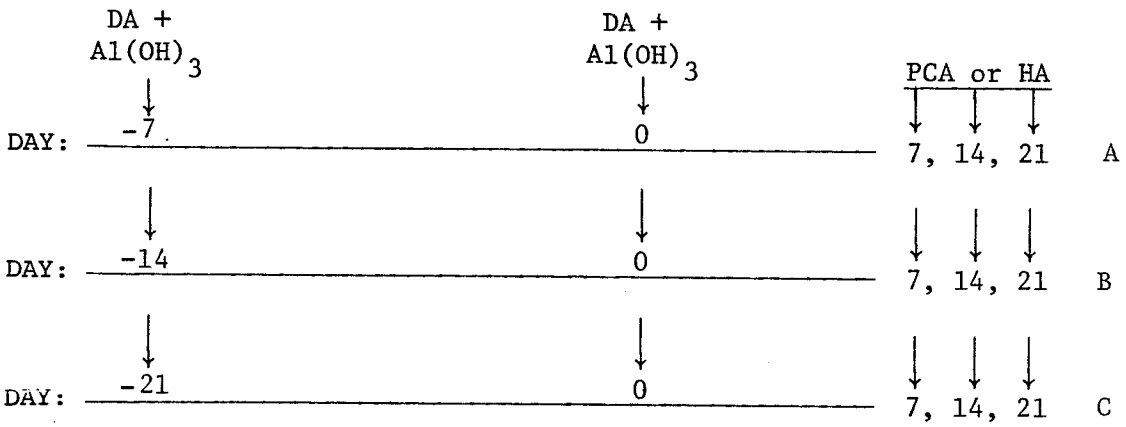


Dose * (μg)	Anti-DA PCA Titers on days		
	14	21	35
.1	<10	<10	<10
1	20	30	1800
10	20	30	1700
100	20	30	80

*Mice were injected i.p. with varying doses of DA in the presence of 1 mg of $Al(OH)_3$. The primary IgE responses were determined on days 14 and 21 after sensitization. The mice were resensitized on day 28 for the elicitation of secondary response measured on day 35.

TABLE XVIII

INCREASE IN SECONDARY IgE RESPONSE
WITH INCREASE IN INTERVAL BETWEEN FIRST AND SECOND SENSITIZATION



DAY	ANTI-DA PCA TITERS		
	A	B	C
7	640	2,500	2,560
14	480	640	2,560
21	160	1,280	2,560

DAY	ANTI-DA HA TITERS		
	A	B	C
7	128	512	4,096
14	256	1,024	4,096
21	512	1,024	4,096

The mice were injected with 1 μg of DA incorporated in $Al(OH)_3$ on days -21, -14 or -7 and resensitized on day 0. The anti-DA IgE and H.A. responses were determined on days 7, 14 and 21.

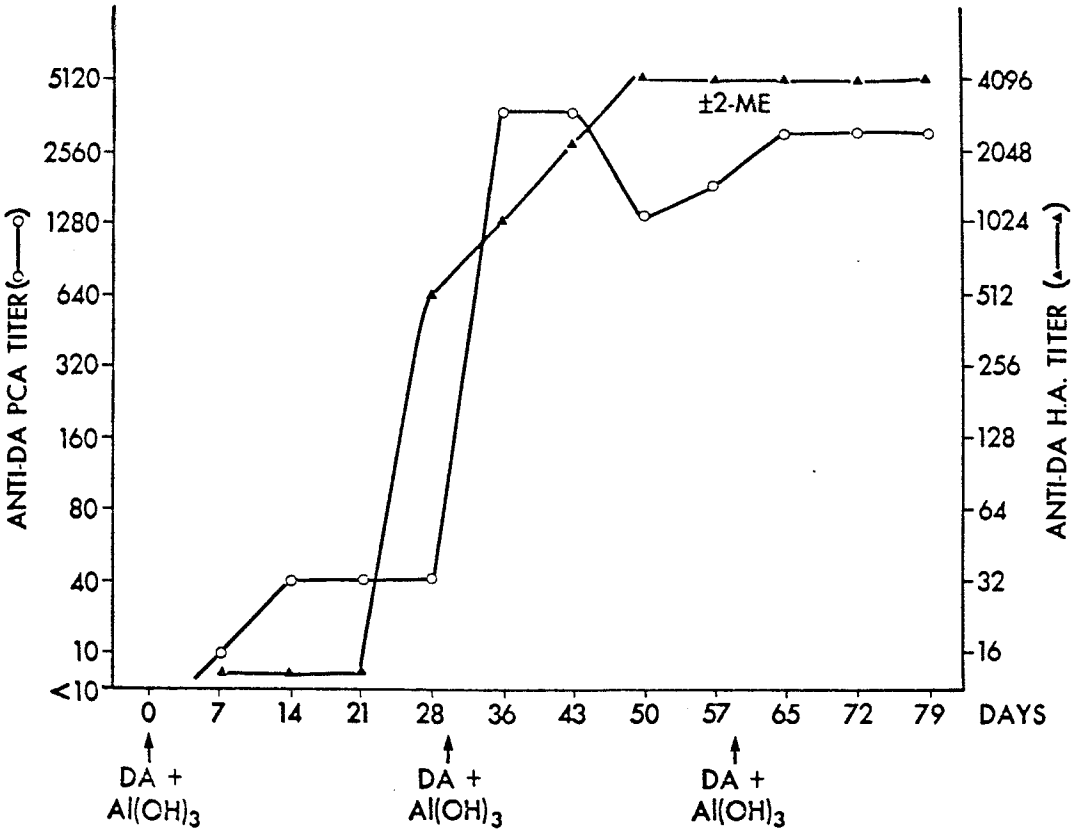


Figure 12: Kinetics of anti-DA IgE and H.A. antibody responses. Normal $B_6D_2F_1$ were immunized with 1 µg of DA incorporated in 1 mg of $Al(OH)_3$ on days 0, 29, and 58. The anti-DA IgE (o—o) and H.A. (▲—▲) antibodies were measured. The hemagglutination assay was also performed in the presence of 2-ME.

and H.A. antibody responses. The H.A. titers in the presence of 2-ME, were identical to the H.A. titers in the absence of 2-ME hence, the class of hemagglutinating antibody was considered to be predominantly IgG.

B) SUPPRESSION OF THE ANTI-DA PRIMARY AND SECONDARY IgE AND H.A. ANTIBODY RESPONSES:

The induction of anti-DA IgE and IgG antibodies were documented in the previous section. The effect of soluble DA on the capacity of mice to mount primary and or secondary anti-DA reaginic responses was determined in the next series of experiments. For this purpose, as reported in Figures 13 and 14, normal mice were administered, on day -1, soluble DA followed by sensitizing doses of DA in $Al(OH)_3$ on days 0 and 29. Primary and secondary anti-DA IgE and H.A. antibody responses were elicited in mice not treated with the soluble DA. However, the primary IgE and H.A. antibody responses of mice, receiving .01, .1, 1, 2 or 4 mg of soluble DA, prior to their sensitization with DA in $Al(OH)_3$ were completely suppressed. Moreover, mice injected with 0.01 or 0.1 mg of soluble DA produced moderate IgE and H.A. secondary responses upon resensitization with DA in $Al(OH)_3$ but, the responses remained significantly lower in magnitude as compared to those from mice injected with saline (Figure 13,14). Furthermore, the secondary anti-DA IgE response of mice pretreated with 1, 2 or 4 mg of soluble DA, was not significantly elevated when challenged with the second sensitizing dose of DA in $Al(OH)_3$ (Figure 13) whereas, the H.A. response was boosted but remained significantly lower as compared to the response of mice injected with saline (Figure 14).

The effect of soluble DA on the responses of mice presensitized with DA in $Al(OH)_3$ was evaluated in the next experiment. The IgE and H.A. responses of mice primed on day -28 with DA in $Al(OH)_3$ were readily abrogated by the administration of soluble DA. The recipients were treated with

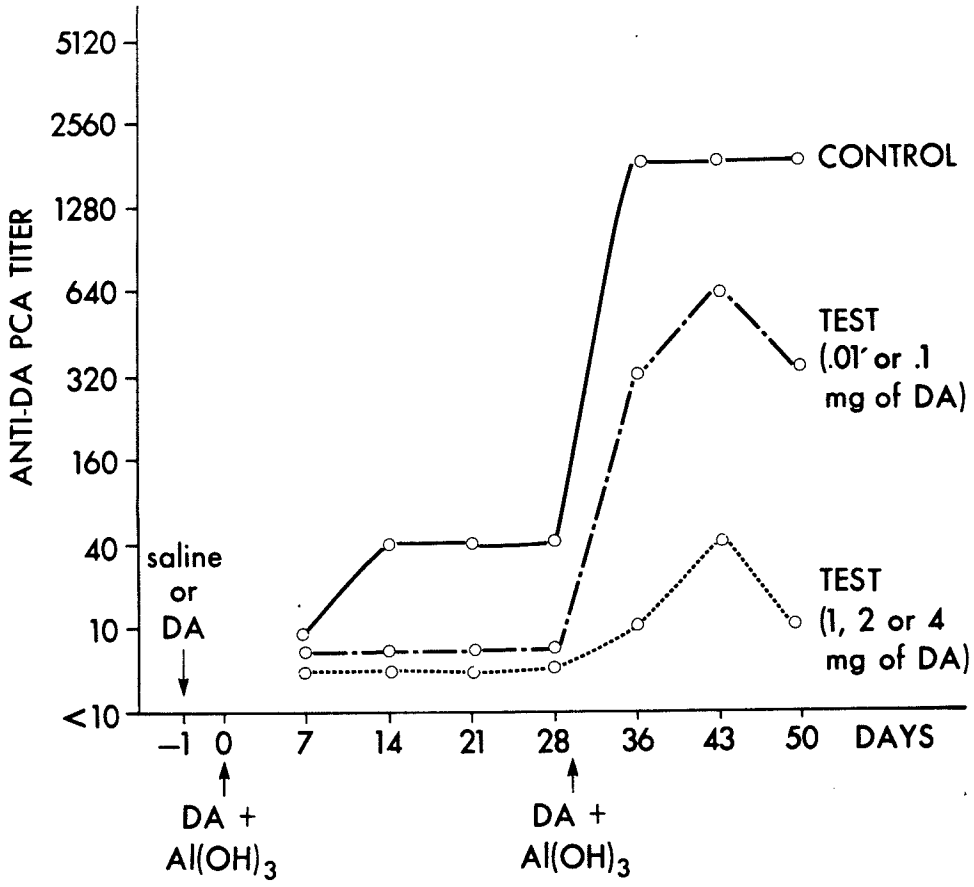


Figure 13: Suppression of the primary anti-DA IgE response.

Normal $B_6D_2F_1$ mice were administered 0.01 or 0.1 mg (o—·—·—o), 1, 2 or 4 mg (o·····o) of DA or saline (o——o) on day -1 and immunized on days 0 and 29 with sensitizing doses of DA.

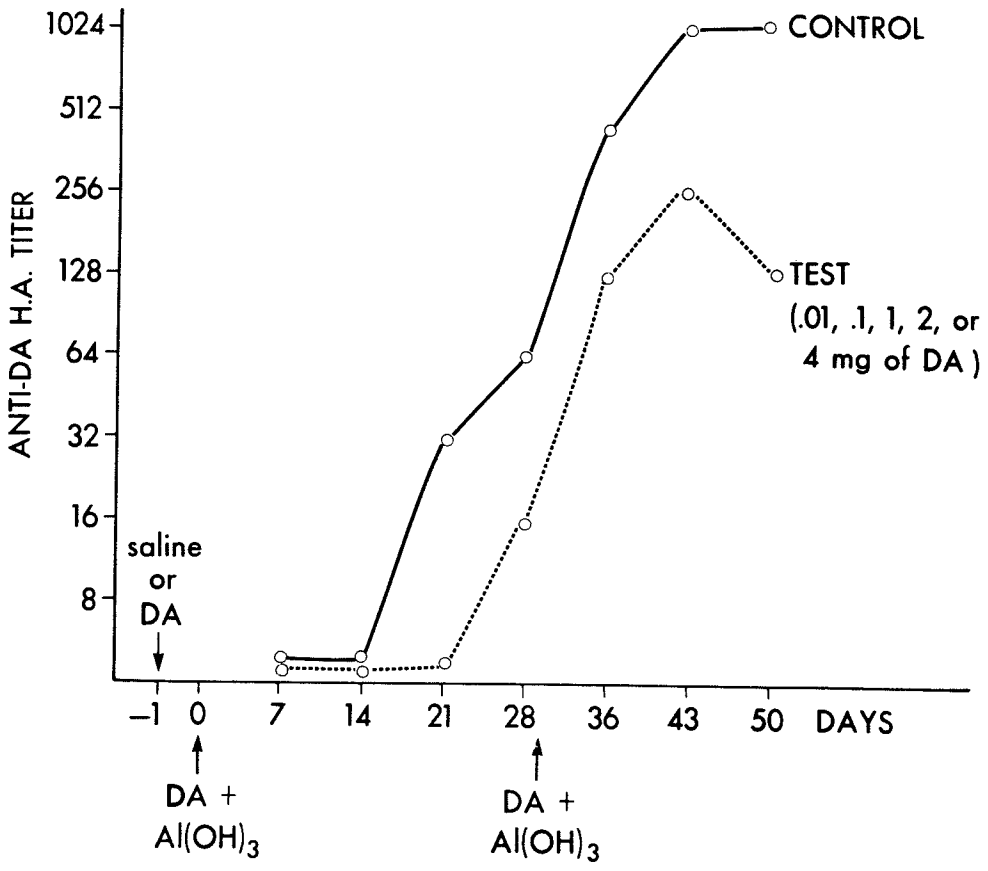


Figure 14: Suppression of the primary anti-DA H.A. responses.

The protocol of this experiment is identical to that of Figure 13.

soluble DA on day -1 and reimmunized on day 0 (Figures 15 and 16). The secondary anti-DA IgE and H.A. antibody responses of these mice were depressed. However, resensitization of the animals with DA in $Al(OH)_3$ on day 29 enhanced the tertiary IgE response of mice treated with 0.01 and 0.1 mg of soluble DA but this response was not prolonged and within 7 days, had dropped by 50% (Figure 15). The IgE antibody response of mice receiving 1,2 or 4 mg of soluble DA were not boosted by the third sensitizing dose of DA in $Al(OH)_3$ given on day 29 (Figure 15). Moreover, the tertiary anti-DA H.A. response of mice receiving saline was increased by the third sensitizing dose of DA in $Al(OH)_3$ given on day 29. However, the response of treated mice remained significantly depressed (Figure 16).

Furthermore, to assure that the suppression of anti-DA H.A. antibody responses was not due to neutralization of circulating antibody, the plaque forming cell response to DA was measured. Two groups of eight mice immunized with DA in $Al(OH)_3$ on day -28 were administered either soluble DA or saline on day -1 (A,B, Table XIX). Four mice from each group were reimmunized with DA in $Al(OH)_3$ on day 0 (group a,c Table XIX) and the anti-DA PFC response of all 4 groups, were measured seven days later using a modified Jerne assay (162). As illustrated in Table XIX, administering soluble DA to mice abolished the anti-DA PFC response (group d) and prevented the development of a secondary anti-DA PFC response (group c). Furthermore, mice sensitized with DA produced mainly indirect plaque forming cells (group a,b), confirming previous results in which the class of antibody detected by hemagglutination was considered to be predominantly IgG (Figure 12).

C) CLEARANCE AND DISTRIBUTION OF SOLUBLE ^{125}I-DA IN MICE PRIMED WITH DA:

The half-life of ^{125}I-DA in the body of mice was calculated as 1 day from the data plotted in Figure 17. Low levels of soluble DA were

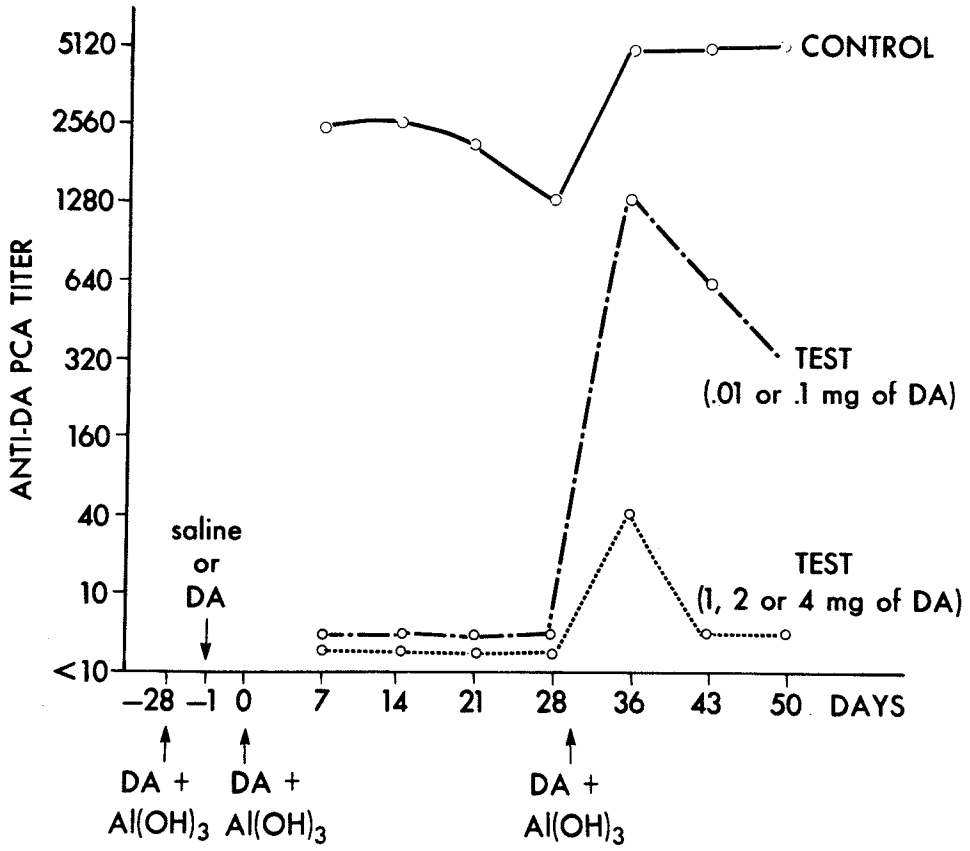


Figure 15: Suppression of the secondary and tertiary IgE response.

Mice, which had been immunized with DA 27 days earlier, were treated with saline (o——o) or with 0.01, 0.1 (o–·–·–o), 1,2 or 4 mg (o·····o) of soluble DA on day -1 and resensitized on days 0 and 29 with DA and $Al(OH)_3$.

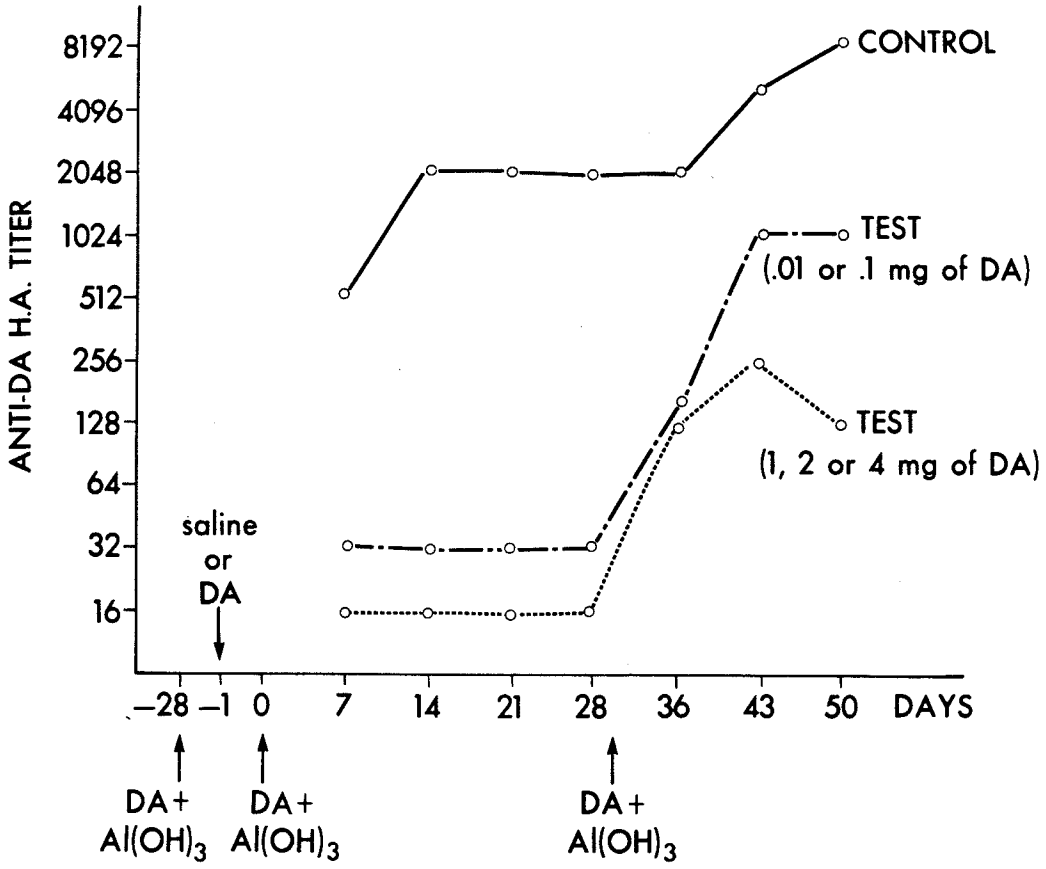


Figure 16: Suppression of the secondary and tertiary H.A. antibody responses. The protocol of this experiment is identical to that of Figure 15.

TABLE XIX

SUPPRESSION OF ANTI-DA PLAQUE-FORMING CELLS RESPONSE BY SOLUBLE DA

		DA + $Al(OH)_3$ Day (-28)	DA (2 mg) Day (-1)	DA + $Al(OH)_3$ Day (0)	PFC/SPLEEN Direct	Indirect
A	a)	+	-	+	400	13,400
	b)	+	-	-	0	200
B	c)	+	+	+	0	400
	d)	+	+	-	0	0

All mice were presensitized with DA + $Al(OH)_3$ on day -28 (group A,B). Two groups of mice were treated either with soluble DA (B) or with saline (A) on day -1. Groups a), c) were resensitized with DA + $Al(OH)_3$ on day 0 whereas group b) and d) were untreated. Direct and indirect anti-DA PFC/spleen were measured on day 7.

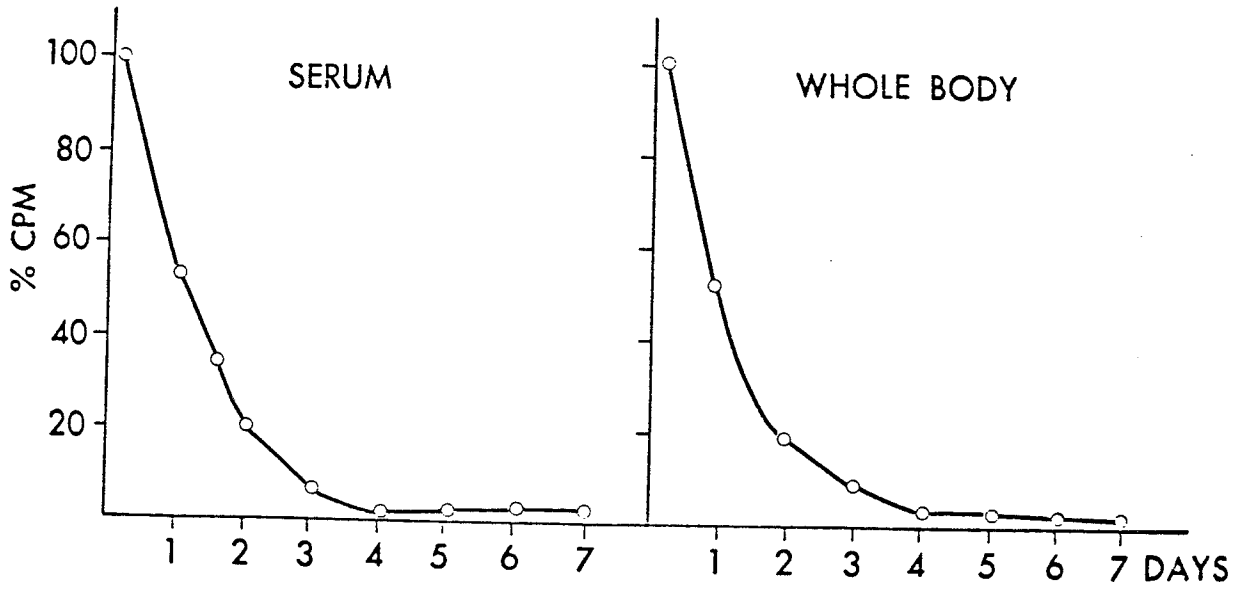


Figure 17: Clearance of ^{125}I-DA

Radiolabelled ^{125}I-DA (2 mg) (with radioactivity of 1.0×10^7 cpm (serum) or 1.0×10^6 cpm (whole body))was injected into mice, primed with DA on day -27 and reimmunized on day +1 with DA in $Al(OH)_3$. Sample of whole blood (25 ul) and the whole body were measured at regular intervals in a gamma counter.

detected in the body 12 days after the injection of ^{125}I-DA (results not shown), by contrast, serum levels of ^{125}I-DA were undetectable after 7 days.

The distribution of soluble ^{125}I-DA among different organs, 12 days after its administration is shown in Table XX. Thus, it appears that soluble DA was preferentially taken up by kidney, liver and intestines and that no significant amount of soluble DA was taken up by the thymus, heart and spleen.

D) SPECIFICITY OF IMMUNOSUPPRESSION WITH SOLUBLE DA

The specificity of the immunosuppressive effect of soluble DA was tested as illustrated by the protocol given in Table XXI. Two groups of 8 normal mice were administered DA or saline. Both the test and control groups were then subdivided into two groups of 4 mice and all animals received a sensitizing dose of DA or OA in $Al(OH)_3$ on day 0 and 29, (Table XXI). As is obvious from the results listed in the lower section of Table XXI, treatment of normal mice with soluble DA did not interfere with the subsequent primary and secondary anti-OA responses which were elicited by sensitization with OA in the presence of $Al(OH)_3$. On the other hand, both the primary and secondary anti-DA IgE responses of mice treated with soluble DA were suppressed when these mice were immunized with the sensitizing dose of DA in $Al(OH)_3$.

Furthermore, the specificity of the suppression by soluble DA on the ongoing response was also tested as reported in Table XXII. Mice primed with DA in $Al(OH)_3$ on day -28 were injected with soluble DA or saline on day -1 and immunized on day 0 with DA or OA in $Al(OH)_3$. A secondary anti-DA response was produced in mice treated with saline and immunized with DA in $Al(OH)_3$, however, mice injected with soluble DA

TABLE XX

RESIDUAL ^{125}I-DA IN DIFFERENT ORGANS ON DAY 12

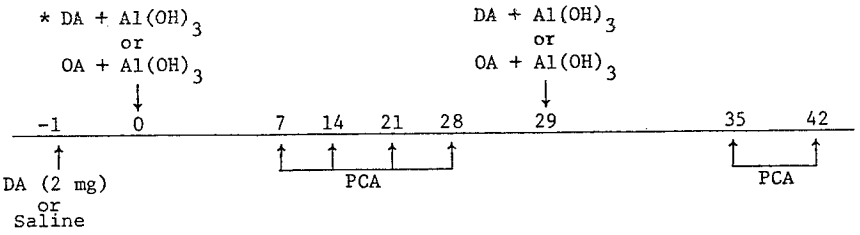
ORGANS	% CPM
Thymus	.02
Heart	.14
Kidney	2.23
Spleen	.03
Liver	2.7
Intestine	15.4

$$\% \text{ CPM} = \frac{\text{CPM of organ}}{\text{CPM of whole body}} \times 100\%$$

Mice received 2 mg of ^{125}I-labelled soluble DA with an original specific activity of 1.0×10^7 C.P.M. After 12 days, the mice were sacrificed and the total radioactivity in the above organs was determined.

TABLE XXI

SPECIFICITY OF SOLUBLE DA-INDUCED SUPPRESSION IN NORMAL $B_6D_2F_1$ MICE

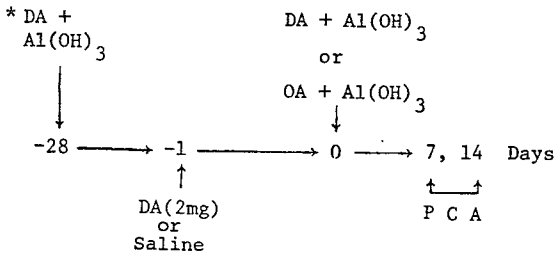


	DAYS	PCA TITERS			
		DA in $Al(OH)_3$		OA in $Al(OH)_3$	
		ANTI-OA	ANTI-DA	ANTI-OA	ANTI-DA
CONTROL GROUP					
Saline	-1				
Immunized	0				
Bleeding	7	<10	<10	<10	<10
	14	<10	<40	1,280	<10
	21	<10	<25	640	<10
	28	<10	40	640	<10
Immunized	29				
Bleeding	35	<10	2,560	2,560	<10
	42	<10	2,560	2,560	<10
TEST GROUP					
2 mg of DA	-1				
Immunized	0				
Bleeding	7	<10	<10	<10	<10
	14	<10	<10	1,280	<10
	21	<10	<10	640	<10
	28	<10	<10	640	<10
Immunized	29				
Bleeding	35	<10	40	2,560	<10
	42	<10	<10	2,560	<10

* All mice received sensitizing doses of DA or OA in $Al(OH)_3$ on day 0 or 29. The test mice received an additional injection of 2 mg of soluble DA on day -1.

TABLE XXII

SPECIFICITY OF SOLUBLE DA-INDUCED SUPPRESSION IN MICE PRIMED WITH DA



P C A TITERS

		DA + $Al(OH)_3$		OA + $Al(OH)_3$	
	DAYS	ANTI-OA	ANTI-DA	ANTI-OA	ANTI-DA
CONTROL GROUP					
Primed with DA	-28				
Saline	- 1				
Immunized	0				
Bleeding	7	<10	2560	<10	1280
	14	<10	1280	1280	1280
TEST GROUP					
Primed with DA	-28				
2mg of DA	- 1				
Immunized	0				
Bleeding	7	<10	<10	<10	<10
	14	<10	<10	1280	<10

* All mice were presensitized with DA on day -28 and resensitized on day 0 with either DA or OA in $Al(OH)_3$. The test mice were injected with 2 mg of soluble DA on day -1.

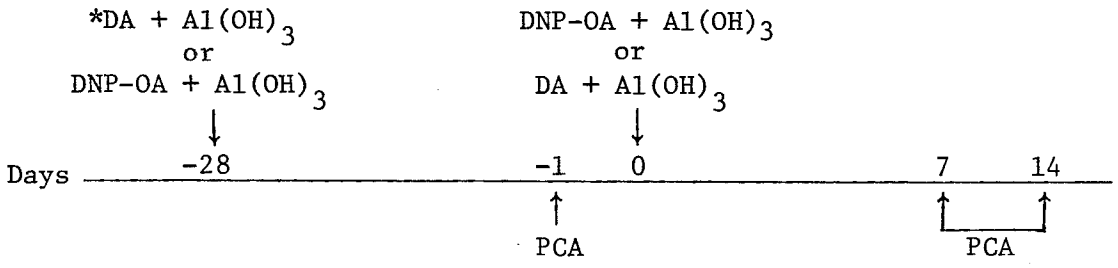
did not produce any secondary response. No anti-OA IgE antibodies were detected in these two groups of mice. In addition, mice primed with DA on day -28 and reimmunized with OA in $Al(OH)_3$ previously administered saline or soluble DA, mounted a primary anti-OA response. Moveover, a secondary anti-DA response was detected in the group of mice primed with DA in $Al(OH)_3$ on day -28 and resensitized with OA in $Al(OH)_3$. A similar finding was observed when α-A was used in place of OA as the sensitizing antigen.

E) DETERMINATION OF ANTIGENIC CROSS-REACTIVITY BETWEEN OA AND DA

As shown in the previous section, a secondary anti-DA IgE response was produced by mice primed with DA and immunized with OA or α -A in $Al(OH)_3$. In order to determine if DA and OA shared similar antigenic determinants, the following experiment was performed (Table XXIII). Each of 4 groups of mice primed with DA or with DNP-OA in $Al(OH)_3$ were bled 1 day prior to their sensitization with DA, or DNP-OA in $Al(OH)_3$. Mice, primed and resensitized with DNP-OA in $Al(OH)_3$ produced a secondary anti-OA and anti-DNP IgE response and had a slightly elevated anti-DA IgE response (Table XXIII, group 3). On the other hand, mice primed with DNP-OA in $Al(OH)_3$ and challenged with DA in $Al(OH)_3$ did not boost either the anti-OA, anti-DNP or anti-DA IgE responses (group 4). However, immunizing mice, primed with DA in $Al(OH)_3$, with DNP-OA in $Al(OH)_3$ enhanced the anti-DA IgE response and produced primary anti-OA and anti-DNP reaginic responses (group 1). Moreover, anti-OA and anti-DNP IgE responses were not elicited by resensitizing these mice with a second dose of DA in $Al(OH)_3$ but a secondary anti-DA reaginic response was produced (group 2).

TABLE XXIII

DETERMINATION OF B CELL CROSSREACTIVITY BETWEEN DA AND OA



		PCA TITERS		
DA PRIMED MICE	DAYS	ANTI-OA	ANTI-DNP	ANTI-DA
1) bleeding	-1	<10	<10	40
DNP-OA in $Al(OH)_3$	0			
bleeding	7	<10	<10	1,280
	14	160	640	640
2) bleeding	-1	<10	<10	40
DA in $Al(OH)_3$	0			
bleeding	7	<10	<10	1,280
	14	<10	<10	2,500
DNP-OA PRIMED MICE				
3) bleeding	-1	2,560	640	10
DNP-OA in $Al(OH)_3$	0			
bleeding	7	5,120	2,560	160
	14	10,240	2,560	160
4) bleeding	-1	1,280	640	40
DA in $Al(OH)_3$	0			
bleeding	7	2,560	640	40
	14	640	160	200

* Mice were presensitized with either DA (groups 1, 2) or DNP-OA (groups 3, 4) in $Al(OH)_3$ on day -28, bled on day -1 and immunized with DA (groups 2,4) or DNP-OA (group 1,3) in $Al(OH)_3$ on day 0.

These results suggested a possible one way cross-reaction between DA and OA. This hypothesis was assessed by the neutralization of anti-DA and anti-OA PCA activity by soluble DA and OA. However, the PCA activity of the sera was neutralized only by the antigen which elicited the antibody. Thus, providing evidence, that no cross-reacting B cell determinants existed between DA and OA. In order to determine why mice primed with DA produced a secondary anti-DA IgE response when resensitized with an unrelated antigen in $Al(OH)_3$, the effect of $Al(OH)_3$ only on the induction of secondary anti-DA IgE responses was evaluated. For this purpose, mice primed with DA were injected with $Al(OH)_3$ or saline and bled 7 and 14 days later. A secondary anti-DA reaginic response was elicited in animals injected with $Al(OH)_3$ (PCA titer = 2500) whereas, saline treated mice did not produce such a response (PCA titer = 20). Furthermore, in unpublished observations, Lang and Filion have shown, in rats, an elevation of total IgE levels by the administration of $Al(OH)_3$, indicating the enhancement of nonspecific IgE synthesis.

DISCUSSION

The optimal conditions for the induction of IgE, H.A. and PFC responses to DA are reported in this chapter. A sustained and prolonged anti-DA IgE response was produced in mice by the injection of two sensitizing doses of 1 or 10 µg of DA incorporated in $Al(OH)_3$ given at an interval of at least 21 days. Other doses of antigen were unable to elicit a prolonged response (Tables XVII, XVIII). The primary anti-DA IgE response was very low and the involvement of non-specific T suppressor cells as described by Katz (163), in dampening reaginic responses was investigated. The effect of cyclophosphamide and low doses of irradiation on the primary anti-DA IgE response was minimal (results not shown). It is possible that the treatments were ineffective when employing high responder $B_6D_2F_1$ mice, since these treatments were used effectively with low and moderate responder mice. However, as shown by Drs. Lee and Schwenk (105) of this department, cyclophosphamide or low doses of irradiation eliminated suppressor T cells which permitted the development or the enhancement of the primary anti-OA IgE response in $B_6D_2F_1$ mice. Hence, even though the results are circumstantial, it was concluded that the lack of a primary anti-DA IgE response after the treatment of mice with cyclophosphamide and low doses of irradiation was not due to nonspecific Ts cells and their role in this system was minimal. Therefore it is postulated that the lack of a primary anti-DA IgE response was probably due to inefficient antigen presentation by macrophages or the lack of T helper cell priming.

Furthermore, the results obtained in the timing of the second injection of DA incorporated in $Al(OH)_3$, (Table XVIII) demonstrated that B cells were activated, since with longer periods of time between injections, the minimum being 21 days, sustained anti-DA

reaginic responses were elicited. This interpretation is consistent with the fact that B cells are primed much slower than T cells (164, 165). In addition, the stimulation of anti-DA IgE responses with $Al(OH)_3$ in mice primed with DA in $Al(OH)_3$ indicated that the adjuvant nonspecifically activated the anti-DA IgE response probably through the action of macrophages. The cellular mechanism of activation of anti-DA IgE responses by $Al(OH)_3$ was not elucidated and it is difficult from these results to postulate or explain the cellular mechanism(s) which were involved.

The boosting effect of the anti-DA IgE response of mice which had been primed with DA, either by a sensitizing dose of OA or α -A, was interpreted to indicate a non-specific stimulation of the IgE response by $Al(OH)_3$. No cross-reactivity at the B cell level was demonstrated (Table XXIII) since no secondary anti-OA response was elicited in mice primed with DNP-OA in $Al(OH)_3$ and sensitized with DA and $Al(OH)_3$ whereas a secondary anti-DA response was detected when mice primed with DA in $Al(OH)_3$ were immunized with OA incorporated in $Al(OH)_3$. Furthermore, DA and OA neutralized PCA activity of their respective IgE sera, i.e. DA neutralized anti-DA IgE antibodies and OA neutralized anti-OA IgE antibodies, respectively (results not shown). Moreover, a secondary anti-DA IgE response was elicited in mice primed with DA and treated with 1 mg of $Al(OH)_3$. These results confirm the hypotheses that the secondary anti-DA IgE response detected in mice primed with DA and resensitized by OA or α-A and $Al(OH)_3$ was not due to an antigen cross-reacting at the B cell level (Table XXII). However, experiments detailing the non-specific stimulation of the anti-DA response, taking into consideration a possible cross-reacting T helper cell and the $Al(OH)_3$ effects, must be performed before a final conclusion

can be drawn.

The kinetics of anti-DA IgE and H.A. antibody production were almost identical to each other. The anti-DA and anti-OA kinetic curves were similar in appearance. However, the primary anti-OA response was higher in magnitude than the primary anti-DA response. Mice produced also PFC responses to sensitizing doses of DA in $Al(OH)_3$ and the class of antibody measured in the H.A. and PFC response was predominantly IgG (Table XIX, Figure 12).

Suppression of immune responses with soluble antigens has been widely achieved. Thus, Chiller and Weigle (166) injected deaggregated human gamma globulin into mice and induced a long-lasting state of unresponsiveness which persisted for at least 155 days. This state was mediated by the inactivation of both T and B cells. Moreover, Benacerraf and colleagues demonstrated that mice responding to the synthetic antigen GAT could be categorized into responder and nonresponder strains. The unresponsive state to GAT was shown as a result of a complex cellular interaction involved in the stimulation of specific Ts cells (76,167). Responder and nonresponder strains differed only in the balance between the intensity of activation of Th versus Ts cells. GAT injected into responder strains, was presented in an immunogenic form by antigen presenting cells, presumably macrophage, in relation to their Ia molecules which stimulated Th cells. However, Ts cells were stimulated also in responder mice when antigen presentation by macrophage was interfered with, thus, either stimulating Ts cell directly or through a feedback mechanism by Th cells. In nonresponder mice, GAT stimulated Ts predominantly. The actual mechanism of activation of Ts cell in these strains has not yet been eludicated.

Suppression of reaginic responses with soluble antigen has been achieved mainly by feeding normal mice with high concentrations (20 mg) of the soluble antigen (OA) (137,138). Administering soluble antigen via the intragastric route into sensitized mice had no effect on the reaginic response (140). Furthermore, the injection of soluble antigen by the i.p. or i.v. routes suppressed transiently the IgE response (6,10,11), but these antigens were rendered immunosuppressive by coupling nonimmunogenic carriers (PEG, D-GL) onto them (10,11,135).

However, as reported in this study, some antigens in soluble form can suppress reaginic responses. Soluble DA specifically and markedly abrogated the primary, secondary and tertiary anti-DA IgE responses (Tables XXI, XXII, and Figures 13,15). The unresponsive state was long lasting and maintained in the recipients with only one injection of soluble DA even upon subsequent immunizations with DA in $Al(OH)_3$. Soluble DA markedly inhibited also the production of anti-DA H.A. and PFC responses (Table XIX, and Figures 14,16). Thus, the involvement of blocking antibodies in the dampening of reaginic response to DA was not considered as part of the mechanism in inducing the unresponsive state in these mice. Soluble DA was cleared rapidly from the circulation and body with a half-life of approximately 1 day (Figure 17) and the radioactive levels of ^{125}I-DA was maintained in the body for at least an additional 6 days. High levels of radioactivity were measured on days 6 and 12 (Table XX) in the intestines with little or no radioactivity associated with the spleen (data for day 6, not shown).

Although the cellular mechanism of the unresponsive state is discussed in the next chapter, the association of radioactive antigen with the intestines and not the spleen, must be commented on. As described in a later section, the spleen was a rich source of Ts cells. Mattingly

and Waksman (139) demonstrated that Ts cells were induced in the Peyer's patches and migrated to various organs i.e. spleen; it is possible that soluble DA induced the formation of Ts cells in Peyer's patches which migrated to the spleen. Furthermore, antigen was not associated with the spleen cells since radioactive levels in the heart and spleen were similar. However, it is possible that a super-suppressive fragment was formed and was associated with the T cell population of the spleen. Further analysis of the cellular mechanism is discussed in the next chapter.

CHAPTER 4

INTRODUCTION

CELLULAR MECHANISM(S) INVOLVED IN THE SUPPRESSION OF ANTI-DA IgE RESPONSE OF MICE TREATED WITH SOLUBLE DA.

Various investigators studying the regulation of the IgE response over the last few years, have described nonspecific Ts cells which dampened the reaginic antibody response to various antigens. Kishimoto et al (107) demonstrated that injection of conjugates of DNP with mycobacterium (DNP-Tbc) induced DNP-reactive Ts cells, which on exposure to conjugates of DNP even with unrelated carriers, released a suppressor factor capable of abrogating selectively the IgE antibody response not only to DNP, but also to all other determinants. On the other hand, Dessein et al (76), showed that the IgE response to the synthetic polypeptide antigen GAT was under the influence of H-2 linked genes and suppressor cells. The data provided evidence for the simultaneous existence of antigen-specific H-2 linked Ir gene regulation in well as immunoglobulin class-specific regulation of IgE response to a single antigen, and demonstrated the role of irradiation and cyclophosphamide (CY)-sensitive suppressor cells in the regulatory events.

The use of protein antigen in the study of the regulation of the reaginic antibody response was described in the preceding chapter. It was the first demonstration that soluble antigen specifically suppressed the primary and more importantly, the secondary IgE response. The failure of other soluble antigens to induce long lasting suppression of specific reaginic antibody response is probably due to a lack of specific antigenic determinants which stimulate the appropriate cellular pathway that dampens the response to the antigen in question (168).

However, modified antigens which were prepared by conjugation to appropriate non-immunogenic carriers (6, 135) or by denaturation (5) or aggregation (8) specifically activated cellular pathway(s) which abrogated IgE responses.

The cellular mechanism involved in the suppression of the anti-DA IgE response by soluble DA are analyzed in this chapter. It is demonstrated that Ts cells were activated by the injection of soluble DA and the recipients had also an impaired Th cell function. Moreover, a soluble extract from Ts cells abrogated the reaginic antibody response of irradiated recipients injected with spleen cells from mice primed with DA.

MATERIALS AND METHODS

ANIMALS: Inbred 6 to 12-week old (C57BL/6 x DBA/2)F_1 male mice (designated as $B_6D_2F_1$) were purchased from either Timco, Ltd., Houston, Texas or Gunton Vivarium, Gunton, Manitoba and random-bred hooded rats from Bio-Breeding Laboratories Ltd., Ottawa, Ontario. Guinea pigs were obtained from High Oak Ranch, Goodwood, Ontario.

PROTEIN ANTIGENS AND OTHER REAGENTS: Keyhole limpet hemocyanin was purchased from Calbiochem, La Jolla Ca., while agarose, from Miles Lab. Elkhart, In. and monoclonal anti-theta (Thy-1,2) from Olac 1976 Ltd., Bicester, Oxon, England. Rabbit anti-mouse Ig serum and DNP-KLH conjugate were kindly donated by P. Pakzad and R. Schwenk respectively. All other reagents were obtained as described in Chapters 2 and 3. Guinea pig complement was prepared from blood obtained by cardiac puncture of outbred male guinea pigs. The serum (1 ml) was absorbed with agarose (50 mg) at $4^{o}C$ for 1 hour to remove non-specific antibodies to polysaccharides. The mixture was filtered through 4 layers of gauze (mesh size 19 x 15/sq.in.) and the supernatant was centrifuged at 18,000 RPM for 30 min. The non-specific cytotoxicity and the complement activity of the absorbed guinea pig serum were assayed by the use of cells from thymus and spleen and monoclonal anti-theta serum. The complement activity of the serum diluted at 1/4 using monoclonal anti-theta antibody was 20-35% of the spleen cells and 100% for thymus, whereas further dilutions of the complement were not as effective.

IMMUNIZATION AND MEASUREMENTS OF IMMUNE RESPONSES: Induction of optimal murine IgE responses and PCA assays were performed as described in Chapter 2 p. 39 and Chapter 3 p. 100.

CYTOTOXIC KILLING OF SPLENIC LYMPHOCYTES: The enrichment of splenic T and B cells was achieved by differential cytotoxic killing of B and T

cells by the use of rabbit anti-mouse Ig and monoclonal anti-theta (anti-Thy-1,2) respectively, in the presence of guinea pig complement. Briefly, spleens were dissociated into single cell suspensions and the cells were subjected to density centrifugation over Na-metrizoate - Ficoll (1.69). The cells at the interface of medium and Na-metrizoate - Ficoll were collected, washed in MEM, and counted under the microscope. The cell concentration was adjusted to 5×10^7 cells per ml, and the monoclonal anti-theta serum (1/800) (v/v) or rabbit anti-mouse Ig (1/150) (v/v) was added to the cells and incubated at $4^{\circ}C$ for 15 min. Guinea pig complement (1:4) (v/v) was added to the mixture and incubated in a $37^{\circ}C$ water bath for 45 minutes; an aliquot was taken then for the determination of percent cytotoxicity. The remaining mixture was passed over Na-metrizoate-Ficoll and the cells at the interface were collected, washed, counted and injected into the recipients as outlined in the different experiments. The percent cytotoxicity obtained with rabbit anti-mouse Ig and monoclonal anti-theta ranged from 45-55% and 20-35% of the whole spleen, respectively.

RESULTS

A) Suppression of anti-DA IgE and H.A. antibody responses by soluble DA

It was shown previously (chapter 3) that soluble DA specifically suppressed the anti-DA IgE and H.A. antibody responses. Thus, mice primed with DA in $Al(OH)_3$ on day -28 were treated with soluble DA on day -1 and resensitized with DA in $Al(OH)_3$ on day 0 and 29 (Figure 18). The anti-DA IgE and H.A. antibody responses were both suppressed and the unresponsive state was not boosted by the second challenging dose of DA in $Al(OH)_3$ demonstrating that this state was long lasting.

B) Elucidation of the cellular mechanism(s) involved in the suppression of the anti-DA IgE response of mice treated with soluble DA

1. Transfer of spleen cells from mice treated with soluble DA into mice either primed with DA or irradiated (650R).

Cell transfer experiments were performed in order to elucidate the cellular mechanism(s) involved in the suppression of the anti-DA IgE response by soluble DA. Mice either primed with DA in $Al(OH)_3$ on day -35 or irradiated were injected i.p. with spleen cells from donors primed with DA in $Al(OH)_3$ on day -35, treated on day -8 with soluble DA or saline and reimmunized on day -7 with a sensitizing dose of DA in $Al(OH)_3$. The recipients were rechallenged with sensitizing dose of DA in $Al(OH)_3$ on the same day and their PCA titers were measured on the days indicated in the Tables (Tables XXIV and XXV). The anti-DA IgE titers of recipients primed with DA in $Al(OH)_3$ were not affected by the injection of spleen cells from mice either injected with soluble DA or saline (Table XXIV). However, the unresponsive state of mice treated with soluble DA was maintained upon adoptive transfer of their spleen cells (10^7 spleen cells per recipient (PTP)) into irradiated recipients whereas, a significant anti-DA IgE response was produced by irradiated mice injected with 10^7 spleen cells from mice administered saline (PP)

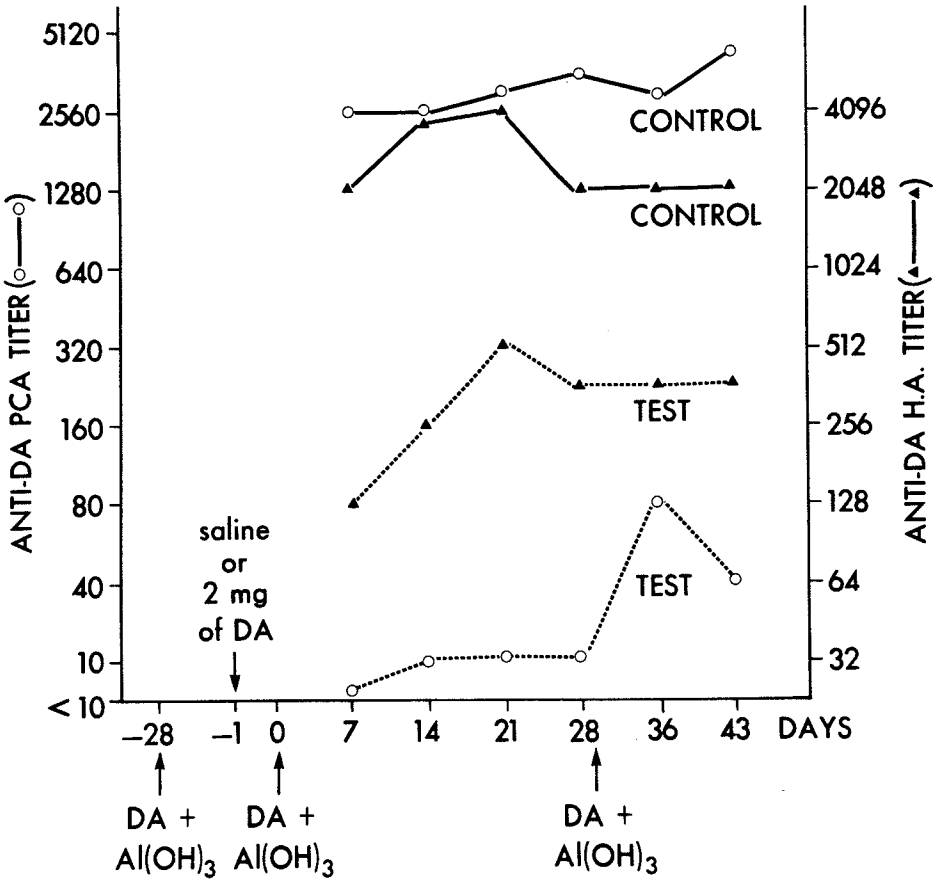
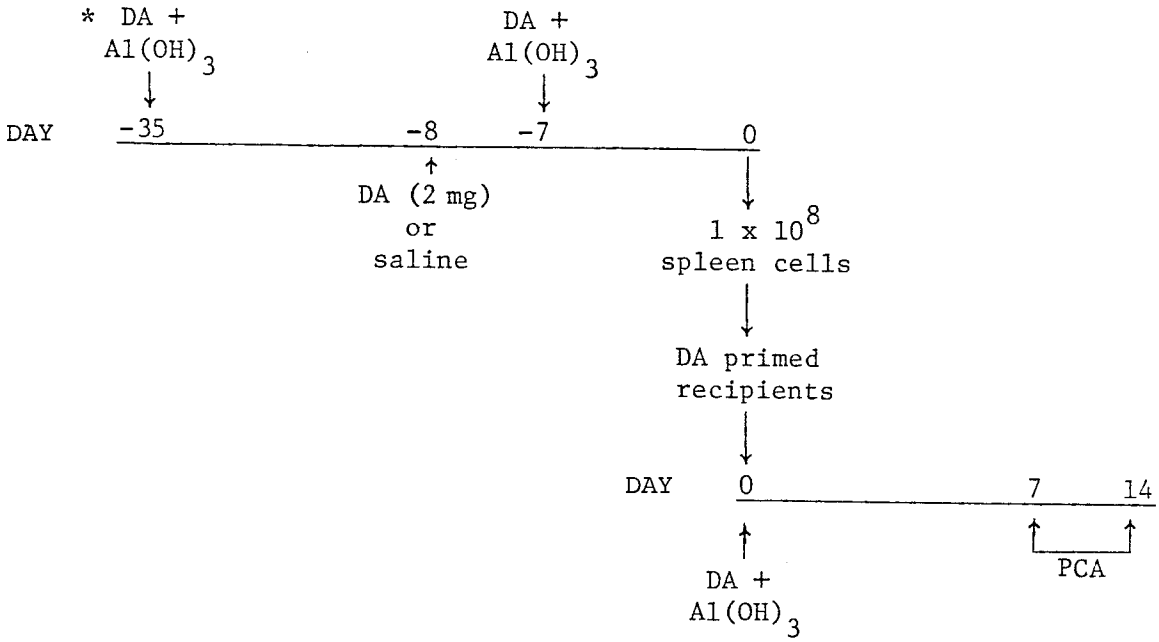


Figure 18: Suppression of the anti-DA IgE and H.A. antibody response by soluble DA.

Two groups of mice, primed 27 days earlier with DA and Al-$(OH)_3$ were either treated with soluble DA or saline on day -1 and immunized with DA and $Al(OH)_3$ on days 0 and 28. The IgE and H.A. antibody titers of test (o·····o, ▲·····▲), and control (o——o, ▲——▲) groups, respectively were measured on days indicated.

TABLE XXIV

FAILURE TO TRANSFER SUPPRESSION INDUCED BY SOLUBLE DA INTO MICE PRIMED WITH DA

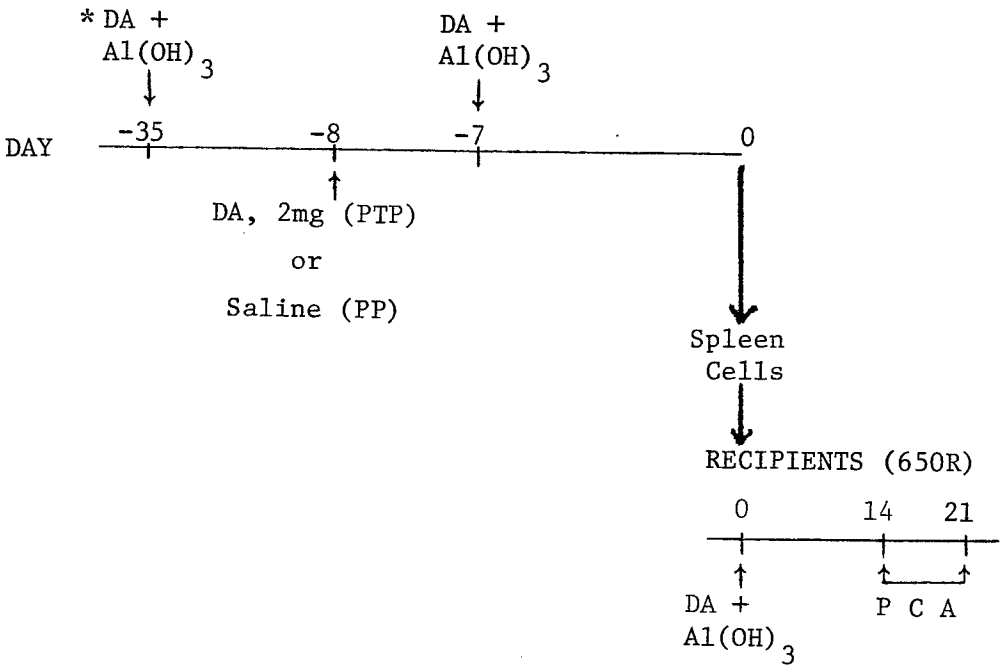


DAY	ANTI-DA PCA TITERS		
	CONTROL GROUP: RECEIVED NO CELLS	RECIPIENTS OF PRIMED CELLS	RECIPIENTS OF SUPPRESSED CELLS
7	2,560	2,560	2,560
14	2,560	2,560	2,560

* Donors of spleen cells, were preimmunized with DA in $Al(OH)_3$ on day -35. Soluble DA (2 mg) or saline was injected into these mice on day -8, followed by a sensitizing dose of DA in $Al(OH)_3$ on day -1. The spleen cells (1×10^8) were transferred on day 0 into recipients presensitized to DA in $Al(OH)_3$ 35 days earlier. A control group received no cells but all three groups of recipients were sensitized with DA in $Al(OH)_3$ on day 0.

TABLE XXV

MAINTENANCE OF SUPPRESSION INDUCED BY SOLUBLE DA IN ADOPTIVE TRANSFER



	ANTI-DA PCA TITERS ON DAYS	
GROUP	14	21
PP	850	1000
PTP	<10	<10

*Donors of spleen cells were preimmunized with DA in $Al(OH)_3$ on day -35, treated with soluble DA (PTP) or saline (PP) on day -8, and resensitized with DA in $Al(OH)_3$ on day -7. Irradiated syngeneic recipients (650R) were injected with spleen cells (10^7) from each group and challenged with DA in $Al(OH)_3$.

2. Effect on T helper cells

The effect of soluble DA on helper T cell function was established in the next experiment. Mice primed with DNP-DA in $Al(OH)_3$ on day -35 were administered soluble DA or saline one day prior to the subsequent rechallenge with the sensitizing dose of DNP-DA in $Al(OH)_3$ on day 0 and 29. The anti-DNP and anti-DA IgE antibody responses of mice treated with soluble DA were considerably reduced as compared to the mice which had received saline (Test groups, Figure 19).

3. Types of cells suppressed by soluble DA

It was reported in the previous sections that the unresponsive state induced by soluble DA could be transferred into irradiated recipients and the injection of soluble DA significantly decreased helper T cell function of the recipients. The next experiments confirmed that T cells were affected by the treatment of mice with soluble DA and determined the effect of soluble DA on B cells of the mice. Thus, syngeneic irradiated recipients were injected on day 0 with various combinations of splenic T and B cells from mice primed with DA in $Al(OH)_3$ on day -35, treated with soluble DA or saline on day -8, and challenged with DA in $Al(OH)_3$ on day -7. The recipients were then immunized with DA in $Al(OH)_3$ (Table XXVI). Irradiated mice injected with enriched populations of B or T cells alone from either suppressed or primed mice produced low levels of anti-DA IgE antibodies, demonstrating that the fractionation of cells into B and T cell populations was successful. Furthermore, recipients of B and T cells from mice administered soluble DA, formed insignificant amounts of anti-DA IgE antibodies: in contrast, recipients of B and T cells from mice, primed with DA in $Al(OH)_3$ produced high levels of anti-DA IgE antibodies. Similar titers were measured in the group of mice primed with DA in $Al(OH)_3$ respectively. However, anti-DA IgE

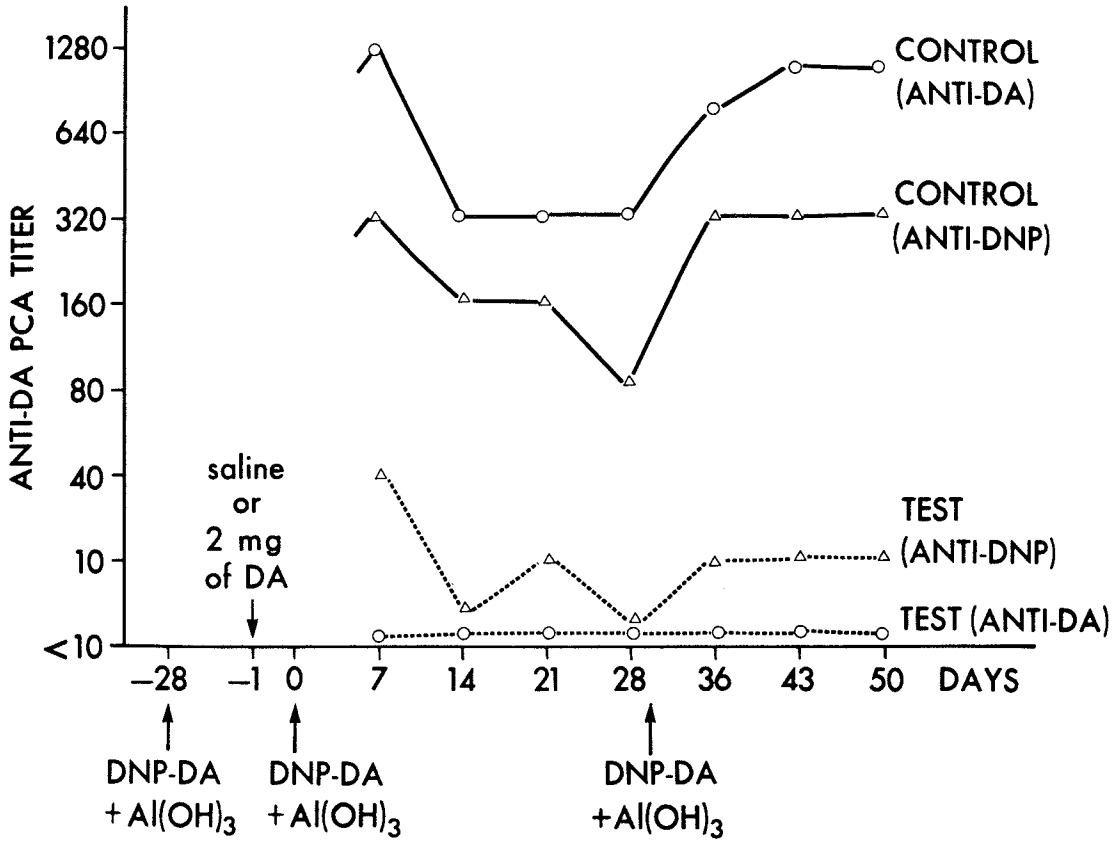
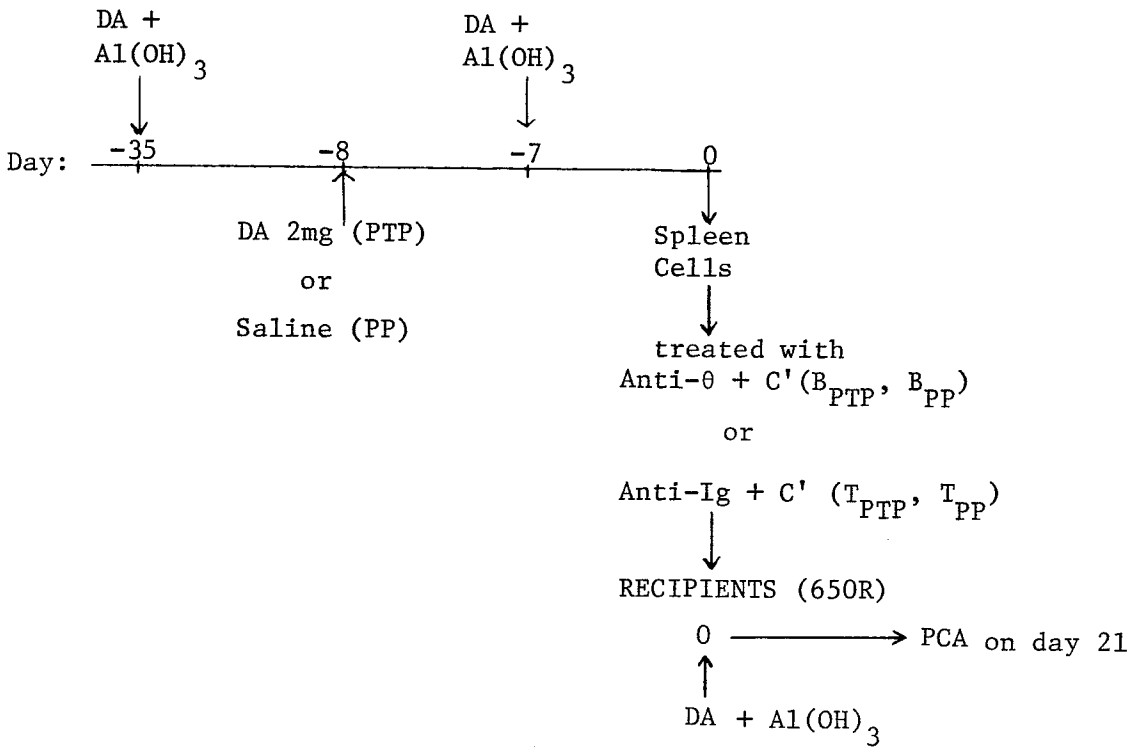


Figure 19: Suppression of the anti-DNP IgE Response: Demonstration of diminished helper function.

Two groups of mice, primed 27 days earlier with DNP-DA and $Al(OH)_3$ were either administered soluble DA or saline on days -1 and resensitized with DNP-DA and $Al(OH)_3$ on days 0 and 28. The anti-DNP, and anti-DA IgE responses of test (Δ·····Δ, O·· ····O) and control (Δ——Δ, O——O) groups, respectively were measured on days indicated.

TABLE XXVI

TYPE OF CELLS SUPPRESSED BY SOLUBLE DA



ANTI-DA PCA TITER (day 21)

GROUPS	TITER	GROUPS	TITER
B_{PTP}	<10	$B_{PTP}+T_{PTP}$	10
T_{PTP}	<10	$B_{PP}+T_{PP}$	320
B_{PP}	<10	$B_{PTP}+T_{PP}$	320
T_{PP}	40	$B_{PP}+T_{PTP}$	<10

* legend next page

TABLE XXVI

TYPE OF CELLS SUPPRESSED BY SOLUBLE DA

*legend

Donors were presensitized with DA in $Al(OH)_3$ on day -35, treated with soluble DA or saline on day -8 and rechallenged with DA on day -7. Spleen cells from both groups were divided into two and treated with anti-θ + C' (B_{PTP}, B_{PP}) or with anti-Ig + C' (T_{PTP}, T_{PP}) and injected in various combinations (1×10^7 B or T cells) into irradiated recipients, followed by a sensitizing dose of DA in $Al(OH)_3$.

antibodies were not detected in recipients of B cells from donors primed with DA and T cells from mice treated with soluble DA.

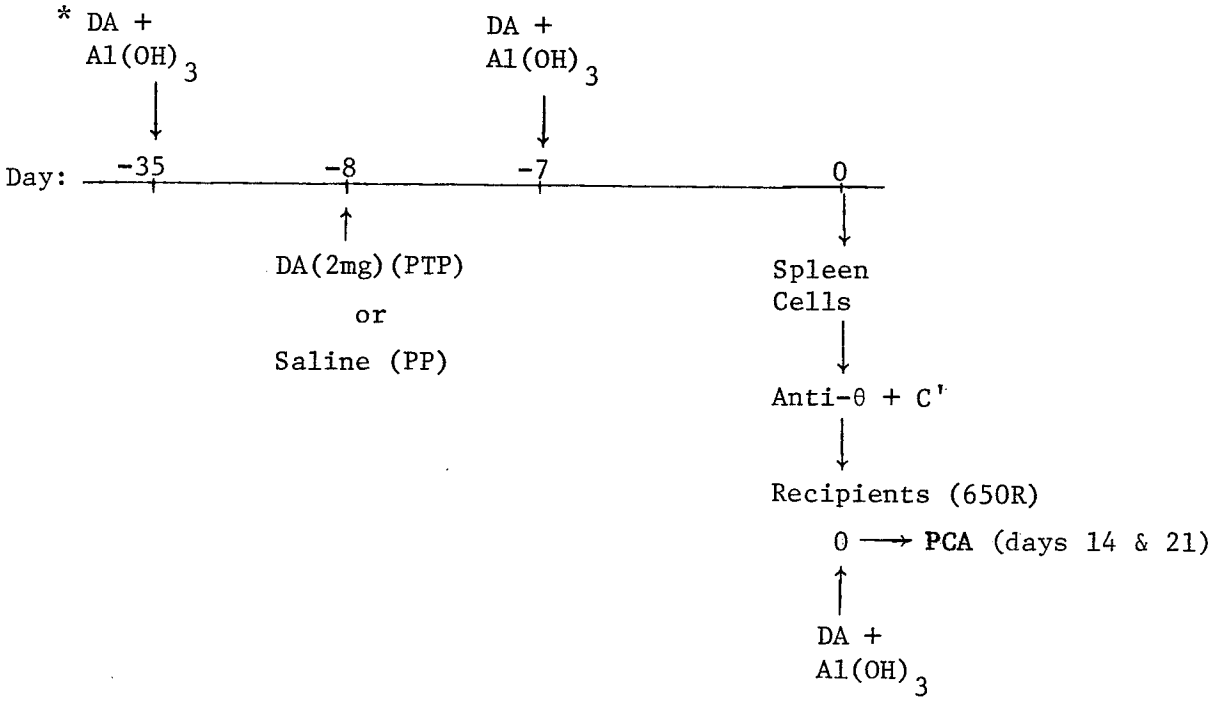
4. Identification of Ts cells

The immunological unresponsiveness achieved by treating mice with soluble DA was maintained by transferring spleen cells from the suppressed mice into irradiated recipients. Moreover, T helper cell function of the treated mice was significantly reduced, whereas B cell function from these mice was not affected. The cellular mechanism involved in the suppression of Th cell function in these animals was analyzed. One may visualize that the dampening effect may be due to an active process such as Ts cells or by a passive process such as clonal abortion or antigen blockade of the receptors on the T cell. Thus, mice primed with DA in $Al(OH)_3$ on day -35 were treated on day -8 with soluble DA (PTP) or saline (PP) and resensitized on day -7 with DA in $Al(OH)_3$ (Table XXVII). Spleen cells from the two groups of mice were injected alone or mixed together into irradiated syngeneic recipients on day 0. Furthermore, spleen cells from group PTP were incubated either with monoclonal anti-theta and complement or complement alone and injected together with spleen cells from group PP . All recipients were challenged with DA in $Al(OH)_3$ on the same day of the cell transfer (Table XXVII). Anti-DA IgE responses were produced by irradiated mice receiving spleen cells of group PP in contrast to mice receiving spleen cells from group PTP which produced a low anti-DA IgE response. Moreover, anti-DA IgE responses were drastically reduced in recipients administered a mixture of spleen cells from groups PTP and PP. However, the elimination of T cells with monoclonal anti-theta and complement from group PTP reversed the suppressive effect exhibited in the recipients. Furthermore, complement alone did not exert any influence on the capacity

TABLE XXVII

IDENTIFICATION OF SUPPRESSOR T CELLS

IN MICE TREATED WITH SOLUBLE DA



GROUPS	ANTI-DA PCA TITERS ON DAYS	
	14	21
PTP	<10	<10
PP	850	1000
PTP+PP	90	100
PTP (C')+PP	230	230
PTP(ANTI-θ+C')+PP	1600	1800

* Donors presensitized with DA in $Al(OH)_3$ on day -35, were treated with soluble DA(PTP) or saline (PP) on day -8, and resensitized with DA in $Al(OH)_3$ on day -7. On day 0, spleen cells from treated mice were incubated with monoclonal anti-θ plus complement or with complement. Irradiated syngeneic recipients were injected either with spleen cells from groups, PTP (5×10^7), PP (1×10^7), PTP + PP ($5 \times 10^7 + 1 \times 10^7$), PTP(C') + PP ($1 \times 10^7 + 1 \times 10^7$) or PTP (anti- +C') + PP($3.25 \times 10^7 + 1 \times 10^7$) and challenged with DA + $Al(OH)_3$.

of spleen cells from group PTP to abrogate the anti-DA IgE response.

C) Characteristics of the Ts cells induced by soluble DA

1. Kinetics of appearance of Ts cells

It has been shown that soluble DA suppressed the ongoing anti-DA IgE response of mice and the unresponsive state was at least partially mediated by Ts cells. Furthermore, T helper cell function was decreased in the treated mice. The following experiment dealt with the kinetics of appearance of Ts cells and the results have provided clues as to the cellular mechanism involved. Thus, for this purpose, groups of mice, primed in DA in $Al(OH)_3$ on day -50 were administered saline on day -8 or soluble DA on days -2, -5, -8 or -22. Each of the 5 groups were sensitized with DA in $Al(OH)_3$ 1 day after their treatment and on day 0, their spleen cells were transferred alone or in various combinations into irradiated syngeneic recipients followed by a sensitizing dose of DA in $Al(OH)_3$ (Figure 20).

Low levels of anti-DA IgE antibodies were formed by irradiated mice receiving spleen cells from donors treated with soluble DA on day -5, -8, or -22 (groups B,C,D, Figure 20), in contrast, recipients of spleen cells from mice either treated with soluble DA on day -1 (group A, Figure 20) or primed with DA on day -7 (group E, Figure 20) produced good responses. Furthermore, recipients of the mixture of spleen cells from mice treated with soluble DA or primed with DA in $Al(OH)_3$ produced either high (group A + E), intermediate (group D + E) or low (groups B + E, C + E) anti-DA IgE responses. The results indicated that suppressor T cells were induced between 2 and 5 days after the injection of soluble DA and waned by day 21.

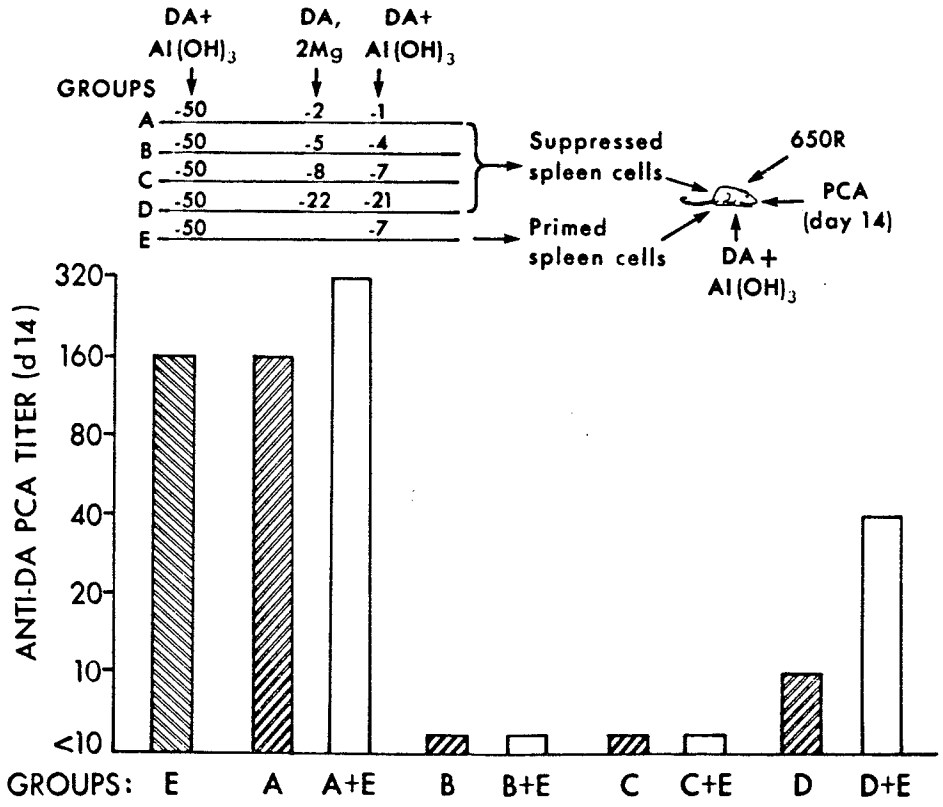


Figure 20: Kinetics of appearance of the T suppressor cells.

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*Figure 20: Kinetics of appearance of the T suppressor cells.

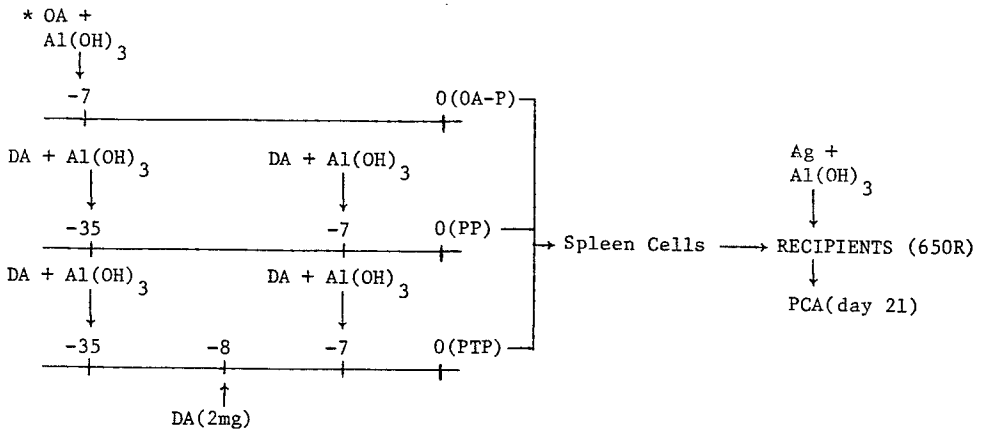
Five groups of mice were primed with DA in $Al(OH)_3$ on day -50. Four of the five groups were injected with soluble DA either on days -2, -5, -8 or -22 and sensitized either on days -1, -4, -7, -21, with DA and $Al(OH)_3$. The fifth group was reimmunized on day -7. On day 0, the spleen cells (1×10^7) were then injected either alone or in various combinations into irradiated syngeneic recipients (650R) and the recipients were challenged with DA and $Al(OH)_3$. All mice were bled on day 14 for the determination of PCA titers.

2. Specificity of the suppressor cells induced by soluble DA

The treatment of mice with a single injection of soluble DA, induced an antigen specific unresponsive state in which Ts cells participated in dampening the anti-DA IgE response. The activation and specificity of the Ts cells induced by soluble DA was investigated by adoptive cell transfers. Spleen cells from three groups of donors, i.e. either from mice immunized with OA in $Al(OH)_3$ on day -7 (OA-P), or from mice primed with DA in $Al(OH)_3$ on day -35 and resensitized with DA in $Al(OH)_3$ on day 7 (PP) or from mice primed with DA in $Al(OH)_3$ on day -35 treated with soluble DA on day -8 and challenged with DA in $Al(OH)_3$ on day -7 (PTP), were transferred in various combinations into irradiated syngeneic recipients and immunized with either DA, OA or a mixture of OA and DA incorporated in $Al(OH)_3$ (Table XXVIII). The challenge of mice with OA and DA will determine if the Ts cell specifically or nonspecifically abrogated IgE responses. The presence of the inducing antigen DA should activate the Ts cell, and their action on the anti-OA IgE response will indicate the specificity of the cells. Hence, the first group of recipients were sensitized with DA in $Al(OH)_3$ only and animals receiving either spleen cells from mice primed with DA or OA in $Al(OH)_3$ produced either a significant or no anti-DA IgE response. Anti-DA IgE antibodies were not detected in recipients of spleen cells from mice treated with soluble DA and anti-OA IgE responses were not elicited in any of the recipients (Table XXVIII). Moreover, irradiated animals were challenged with OA in $Al(OH)_3$ and anti-OA IgE antibodies titers of recipients of spleen cells from mice sensitized with OA were identical even if the recipients received also spleen cells from mice primed with DA or treated with soluble DA. Anti-DA IgE antibodies were not detected in any of the recipients.

TABLE XXVIII

SPECIFICITY OF THE SUPPRESSOR CELLS INDUCED BY SOLUBLE DA



PCA TITERS ON DAY 21

IMMUNIZATIONS	1: DA+Al(OH)$_3$		2: OA+Al(OH)$_3$		3:OA+DA+Al(OH)$_3$	
GROUPS	ANTI-OA	ANTI-DA	ANTI-OA	ANTI-DA	ANTI-OA	ANTI-DA
OA-P	<10	<10	1280	<10	1280	<10
PP	<10	80	<10	<10	<10	80
PTP	<10	<10	<10	<10	<10	<10
OA-P+PP	<10	80	1280	<10	1280	80
OA-P+PTP	<10	<10	1280	<10	1280	<10
PP+PTP	<10	<10	<10	<10	<10	<10

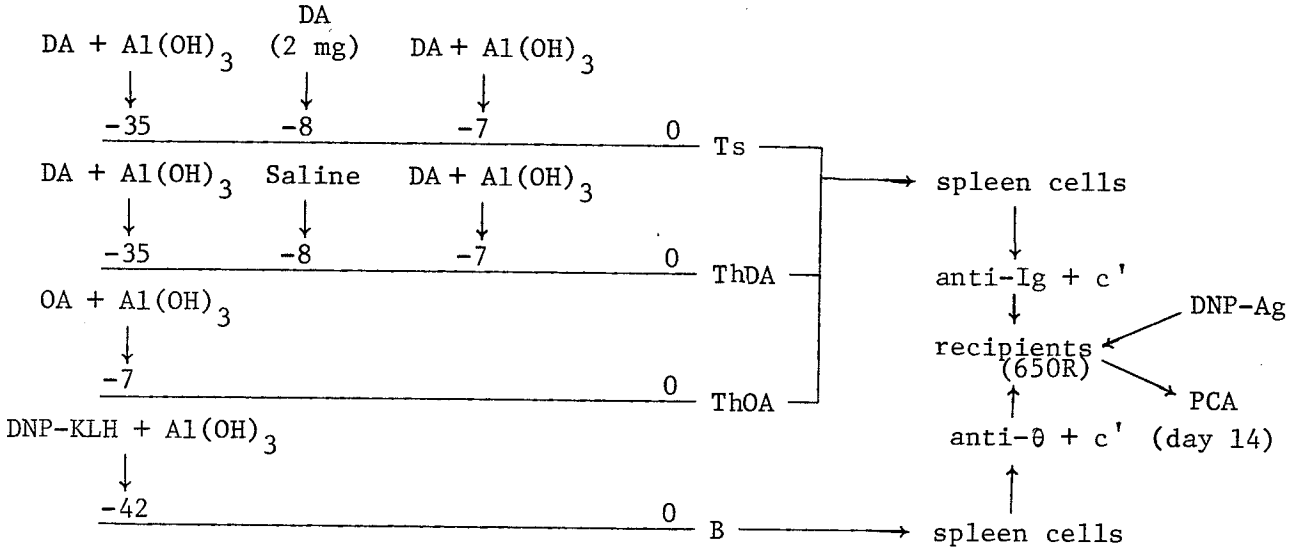
* Donors of spleen cells were sensitized with OA on day -7 (OA-P) or donors presensitized with DA were treated with soluble DA (PTP) or left untreated (PP) on day -8 and resensitized with DA on day 0 and 1 x 10^7 cells from each population was injected in various combinations into irradiated syngeneic recipients . Groups 1, 2 and 3 were then sensitized with DA, OA or a mixture of OA and DA incorporated in Al(OH)$_3$.

The third group of recipient mice were challenged with a mixture of the two antigens, i.e. DA and OA in $Al(OH)_3$. All recipients of spleen cells from mice treated with soluble DA produced negligible anti-DA IgE responses, even if the recipients had received also spleen cells from mice primed with DA or OA in $Al(OH)_3$. However, irradiated mice injected with spleen cells from mice primed with OA in $Al(OH)_3$ elicited a significant anti-OA response even upon receiving spleen cells from mice treated with soluble DA or primed with DA in $Al(OH)_3$. Moreover, spleen cells from mice primed with OA in $Al(OH)_3$ did not affect the anti-DA IgE response of recipients of spleen cells from mice primed with DA in $Al(OH)_3$.

The target of the suppressor cells was determined by employing an anti-hapten response in an adoptive cell transfer system. Mice primed with DA in $Al(OH)_3$ on day -35 were administered soluble DA (Ts) or saline (ThDA) on day -8 and challenged with DA in $Al(OH)_3$ on day -7. Two groups of normal mice were sensitized also with OA in $Al(OH)_3$ (ThOA) on day -7 or DNP-KLH in $Al(OH)_3$ on day -42 (B). The enriched fractions of T or B cells from all 4 groups were obtained as described in Materials and Methods and injected in various combinations into irradiated syngeneic recipients. The recipients were then immunized with 10 µg of DNP-DA, DNP-OA or a mixture of DNP-OA and DNP-DA in saline. As shown in Table XXIX, recipients of Ts, ThDA, ThOA or B cells alone when challenged with DNP-OA in saline produced low levels of anti-DNP IgE, whereas mice receiving either ThDA + B cells or ThOA + B cells elicited anti-DNP reaginic response. Moreover, the anti-DNP response of irradiated animals injected with Ts + ThDA + B cells or Ts + ThOA + B cells was reduced. Furthermore, recipients of Ts, ThDA, ThOA, or B cells and immunized with DNP-OA in saline produced low levels of anti-DNP IgE antibodies. However a significant anti-DNP IgE response

TABLE XXIX

SUPPRESSION OF HELPER FUNCTION WITH SUPPRESSOR T CELLS



IMMUNIZATION	ANTI-DNP PCA TITER (day 14) DNP-DA + SALINE	DNP-OA + SALINE	DNP-OA + DNP-DA + SALINE
Groups			
Ts	<10	<10	<10
ThDA	<10	<10	<10
ThOA	<10	<10	<10
B	80	80	60
ThDA + B	140	<10	240
ThOA + B	1280	640	240
Ts + ThDA + B	30	<10	30
Ts + ThOA + B	160	640	240

*

* Legend see next page

TABLE XXIX

SUPPRESSION OF HELPER FUNCTION WITH SUPPRESSOR T CELLS

* Legend

Donors, presensitized with DA on day -35, were treated with soluble DA (Ts) or saline (ThDA) on day -8 and sensitized with DA in Al-$(OH)_3$ on day -7. Two other groups of donors were immunized with OA in $Al(OH)_3$ (ThOA) or DNP-KLH in $Al(OH)_3$ (B) on day -7 or -42. Enriched fractions of T or B cells were obtained by treating the cells with rabbit anti-Ig + C' or anti-θ + C' as described in Materials and Methods. Irradiated syngeneic recipients were injected with 1×10^7 cells from each population as indicated and sensitized with DNP-DA, DNP-OA or a mixture of DNP-DA + DNP-OA in saline.

was elicited by irradiated mice receiving either ThOA + B cells or more importantly Ts + ThOA + B cells. No detectable anti-DNP IgE antibodies were formed by recipients of ThDA + B cells or Ts + ThDA + B cells suggesting that ThDA did not recognize any antigenic determinants on the OA molecule. In addition, the anti-DNP PCA titers of recipients of Ts, ThDA, ThOA, or B cells and challenged with a mixture of DNP-DA and DNP-OA in saline was low. However, a significant anti-DNP response was detected in irradiated mice injected with either ThDA + B, ThOA + B, or Ts + ThOA + B cells in contrast to mice receiving Ts + ThDA + B cells.

D) Partial characterization of Ts cell factor

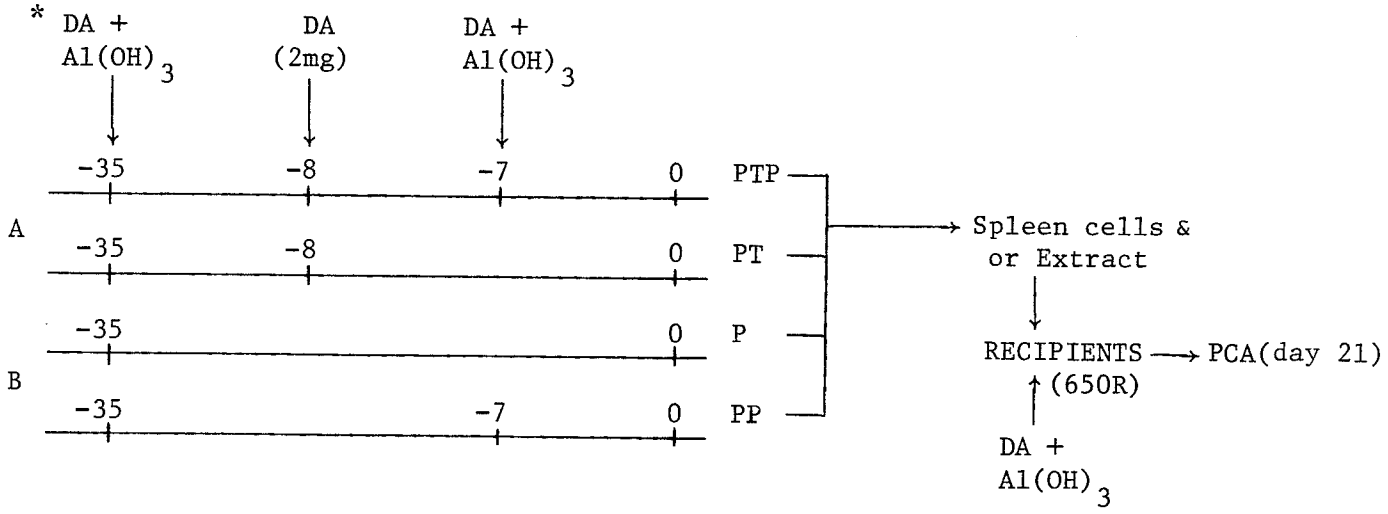
1. Extraction of a soluble factor from Ts cells.

An antigen specific Ts cell was described in the previous section. Tada and associates (180) have demonstrated soluble factor(s) capable of abrogating antigen specific IgE responses in the rat. Thus, experiments were performed in order to determine a soluble factor capable of suppressing reaginic responses, could be extracted from Ts cells induced by soluble DA. Two groups of mice primed with DA in Al-$(OH)_3$ on day -35 were treated with soluble DA (Group A) or saline on day -8 (Group B) and subdivided into two subgroups; one of which being challenged with DA in $Al(OH)_3$ on day -7 (Table XXX).

Spleen cells from groups PT, PTP, and P were divided into two and either incubated with anti-Ig + C' or left untreated. The enriched T cells (5×10^7) from each group were frozen and thawed 3 times, centrifuged at 39,000 x g in a Sorvall RC3 centrifuge for 30 minutes and the supernatant was collected. Irradiated syngeneic recipients were injected either with spleen cells only, mixtures of the various spleen cell populations or spleen cells from mice primed with DA and extracts. The recipients were immunized with DA in

TABLE XXX

DEMONSTRATION OF SUPPRESSOR T CELL FACTOR



	* ANTI-DA PCA TITERS ON DAY 21		
** PTP	<10	PTP+PP***	320
PT	<10	PT+PP	40
P	1280	P+PP	1280
PP	1280	PTP_{EX}+PP	320
		PT_{EX}+PP	160
		P_{EX}+PP	1280

Donors presensitized with DA in $Al(OH)_3$ on day -35 (A and B), were treated with soluble DA (A) or saline (B) on day -8 and subdivided into two subgroups; one of which was challenged with DA in $Al(OH)_3$ on day -7 (PTP and PP). Spleen cells from groups PT, PTP, and P were divided into two and incubated with anti-Ig + C' or left untreated. Cell-free supernatants were obtained from the enriched T cells of each of these groups by freezing and thawing. Irradiated recipients were injected either with the spleen cells only, mixtures of spleen cells, or the spleen cells from mice primed with DA and extracts. All recipients were immunized with DA in $Al(OH)_3$

in $Al(OH)_3$ Table XXI. Mice receiving spleen cells from donors administered soluble DA and either nonimmunized or immunized with DA in $Al(OH)_3$ produced negligible anti-DA IgE responses. However, anti-DA reaginic antibody responses were elicited by recipients of cells from mice primed in DA in $Al(OH)_3$ and/or resensitized with DA in $Al(OH)_3$. Furthermore, the anti-DA IgE response of recipients of spleen cells from mice primed with DA and reimmunized with DA was abrogated by the injection of spleen cells or extracts from mice treated with soluble DA, even if the donors had been reimmunized with DA in $Al(OH)_3$. Spleen cells or extract from mice primed with DA in $Al(OH)_3$ only, had no effect on the anti-DA reaginic antibody response of recipients of cells primed with DA in $Al(OH)_3$ and resensitized with DA in $Al(OH)_3$. The soluble factor extracted from 10^6 cells was still capable of abrogating the anti-DA response of recipients of 10^7 spleen cells from mice primed with DA in $Al(OH)_3$ (results not shown).

2. Molecular weight of Ts cell factor

The apparent molecular weight of the Ts cell factor was determined by fractionation of the extract through a Sephadex G-100 column (2.6 x 100 cm). The extract of the Ts cell was obtained as described in the previous section. As may be seen from the elution profile measured by optical density at 280nm and illustrated in Figure 21, there was only one distinct peak of culture medium, corresponding to the salt peak. Nevertheless, 4 fractions were arbitrarily pooled as indicated in Figure 21, and were concentrated by negative pressure to the original volume of the applied sample and injected into irradiated recipients together with spleen cells from mice primed with DA in $Al(OH)_3$ on days -28 and -7. These mice

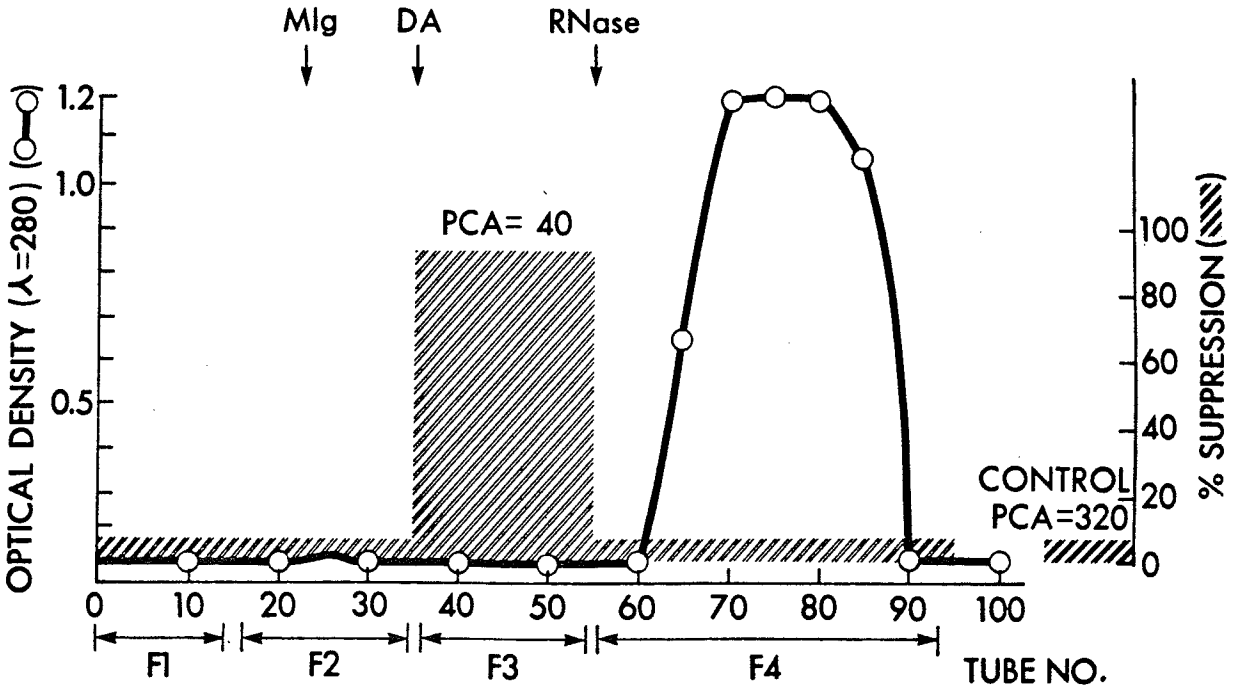


Figure 21: Determination of the molecular weight of Ts cell factor on a Sephadex G-100 column (2.6 x 100 cm)

* legend, next page

Figure 21: Determination of the molecular weight of Ts cell factor on a Sephadex G-100 column (2.6 x 100 cm)

The Ts factor was produced as described in legend of Table XXX and fractionated on a Sephadex G-100 column (2.6 x 100 cm) equilibrated with PBS at pH 7.2, which had been standardized with respect to the elution volumes of mouse IgG, dog albumin, and ribonuclease, as indicated by the arrows. The eluates emerging from the column (o———o) were arbitratily pooled into 4 fractions as indicated, reduced in volume by negative pressure to the sample volume and injected into irradiated recipients with spleen cells (1×10^7) from donors sensitized twice with DA in $Al(OH)_3$ on day -28 and -7. The recipients were then challenged with a sensitizing dose of DA in $Al(OH)_3$ on the day of the transfer (day 0).
The concentration of the factor injected was equivalent to 1×10^7 splenic T cells.

were challenged with DA in $Al(OH)_3$ on day 0 and their IgE antibody titers measured on day 14. As illustrated in Figure 21, the suppressive activity eluted in the molecular weight range of 66,000 to 14,000 daltons. The other fractions had no effect on the anti-DA IgE response.

DISCUSSION

The results in the preceding chapter clealy demonstrated that soluble DA administered to normal mice or mice primed with DA specifically abrogated the primary, secondary and tertiary IgE, H.A. and PFC responses to DA (Figure 18). The unresponsive state, induced with one injection of the soluble antigen, was long lasting and was maintained for at least 2 months even upon reimmunization with antigen and adjuvant.

The cellular mechanism(s) involved in the unresponsive state induced by soluble DA was determined by transferring spleen cells from suppressed mice into mice primed with DA. Thus, transferring 10^8 spleen cells from donors treated with soluble DA into mice primed with DA in $Al(OH)_3$ did not affect the capacity of the recipients to mount anti-DA IgE responses (Table XXIV). The failure to transfer the unresponsiveness into these mice is probably due to the hyper-responsive state of the recipients. If suppressor cells were present, they were unable to prevent the development of the secondary response. The transfer of tolerized cells into normal recipients was not undertaken since only a poor primary anti-DA IgE response was induced in normal mice (Chapter 3). Hence, results from such experiments would be at best only marginal, however, the suppressive state was maintained in irradiated syngeneic recipients (Table XXV). Furthermore, Th cell function of mice suppressed by soluble DA was impaired since anti-DNP IgE responses were not elicited by treated animals when challenged with DNP-DA in $Al(OH)_3$ (Figure 19). Moreover, suppressor T cells were at least partially responsible for the unresponsive state induced by soluble DA, since eliminating T cells with monoclonal anti-theta serum and complement eliminated the dampening effect on the anti-DA IgE response (Table

XXVIII). The unresponsive state in treated animals was possibly due also to the inactivation of B cells. However, it was demonstrated in Table XXVI that B cells of treated mice were not inactivated, or affected since T cells from mice primed with DA were able to provide help to B cells from mice treated with soluble DA and the recipients of these two populations mounted an anti-DA IgE response.

In support of the results reported above, various investigators (161,166,170,171,172,173) achieved antigen-specific suppression of the immune response by injecting large concentrations of soluble antigen (bovine serum albumin or human gamma globulin) and the unresponsive state was mainly due to T cell inactivation with only a small fraction of B cell population, i.e. carrying receptors with a high affinity for the antigen, paralyzed by the treatments. Moreover, T cells were more easily tolerized than B cells, since lower concentrations of antigens could effectively abrogate T cell response. However, B cell population suppressed by the treatments recovered quicker than T cells.

The results in the study of the kinetics of appearance of the suppressor T cell (Figure 20) were similar to those described by Claman and his coworkers (174). Ts cells appeared relatively early in the response (between day 2 and 5) and subsequently waned (day 21). Thus, the role of Ts cells in the long lasting suppression due to the injection of soluble DA has to be re-evaluated. With the disappearance of suppressor T cells, the unresponsive state could be due also to clonal deletion of the Th or amplifier cells (175). However, it is also possible that memory Ts cells were formed, and, with subsequent challenge with antigen, were activated to proliferate and to exert their suppressive effect (105). As an alternative explanation, one may cite the findings of Eardley et al (62), and Cantor et al (176)

who postulated a feedback loop for the regulation of IgG antibody synthesis. In their proposed mechanism, antigen primed Ly-1$^+$ Th cells were shown to act on an intermediate Ly-123$^+$ T_1 cell, so as to generate antigen specific Ly-23$^+$Ts cells which in turn, regulated the activity of Th cell. If a similar feedback mechanism controlled the anti-DA IgE response, soluble DA would prime Th cells which would act on Ly-123$^+$ cells to produce the suppressor Ly-23$^+$ cell resulting in dampening of the response. Upon subsequent immunization with antigen and adjuvant, the feedback loop would be regenerated through the action of Th Ly-1$^+$ cells. Thus, the long lasting suppression induced by soluble DA could be explained. However, in view of a mounting body of evidence, the prolonged dampening of the anti-DA IgE response, could be regulated also by T lymphocytes possessing receptors for idiotypic determinants (177,105). Hence, an alternative mechanism, such as network system, may be involved for the activation and regulation of IgE secreting cells. Moreover, further experimentation is needed in order to determine the cellular pathway involved in the abrogation of the anti-DA IgE response.

The transfer of antigen associated with the spleen cells may explain also the results obtained in the adoptive transfer experiments. However, this hypothesis is unlikely for various reasons. Only a very small amount of radioactive antigen was associated with the spleen 6 or 12 days after the injection of ^{125}I-DA. The possibility exists that a "super suppressive" fragment degraded from DA may be associated with the spleen cells. Nevertheless, spleen cells from mice treated with soluble DA, incubated with anti-theta and complement, washed three times in medium, without any special effort to remove dead T cells were unable to suppress the anti-DA IgE response of irradiated recipients

which had received also spleen cells from mice primed with DA (Table XXVII). The result demonstrated that the suppressive effect was due to live T cells and not to splenic B cells or macrophages. However, it is possible that upon killing Ts cell, antigen associated with the cells is liberated and removed by the washes. To address this question, spleen cells from mice treated with soluble DA on day -2 or -5 were transferred into irradiated mice, together with spleen cells from mice primed with DA (Figure 20). An anti-DA IgE response was induced in the recipients of spleen cells from mice administered soluble DA on day -2 only whereas recipients of spleen cells from mice treated on day -5 did not mount a response. Hence if the suppressive state was due to antigen carry over in the adoptive transfer system, the anti-DA IgE response of recipients of spleen cells from mice injected with soluble DA on day -2 should be abrogated. Therefore, from the results listed above, it was considered unlikely that antigen carry over was involved in the dampening of anti-DA reaginic response in the adoptive transfer system.

The cellular mechanism involved in the induction of high zone tolerance of the IgE response is very similar to results of other investigators (reviewed in 170,172,173,178) who have demonstrated Ts cells in the dampening of the IgG response to the respective antigen in question. However, the mode(s) of action of the cells in the different systems are distinct. Zan-bar et al (175) demonstrated a lack of specific Th cell function in mice treated with BSA, whereas Segal et al (179) showed that suppressor T cells did not impair Th cell function but specifically eliminated or paralyzed antigen-presenting macrophages. The specificity and the target cell of the Ts cell involved in the

dampening of the anti-DA IgE response was elucidated also. The experiments, in which the specificity of the Ts cells was determined, took into consideration the results of Basten et al (160), Kapp et al (107) who demonstrated that Ts could be specifically activated by antigen and exerted a non-specific effect in the presence of a second nonrelated antigen. Thus, employing two different experimental protocols, Ts cells were shown to affect the anti-DA IgE response only and not the response to an unrelated antigen, OA (Tables XXVIII and XXIX). Also the target cells of Ts cells were demonstrated as the T cells, probably Th cells (Table XXIX). If the target cells were B cells or macrophages, then challenging the recipients of Ts + ThOA + B_{DNP} cells with DNP-DA + DNP-OA, would have resulted in a poor anti-DNP IgE response, since suppressive signals from the Ts cell activated by DNP-DA would block all Th cells messages from reaching and activating B cells or the Ts cells would have eliminated the antigen presenting cells. Since a significant response was produced by these recipients, the conclusion that the target cells for Ts cells were Th cells was drawn. However, it is not known if the Th cell are inactivated or eliminated by the Ts cells. Further experimentation with the aid of anti-Ly serum will establish firmly the actual cellular mechanism involved and phenotype of each cell population.

Previously Tada (180) and Kapp et al (59), either by freezing and thawing or sonicating Ts cells, were able to extract a soluble suppressor factor capable of dampening the response to the antigen. An identical observation was made in the present study in which a suppressor factor was extracted from spleen cells of mice administered

soluble DA. On the other hand, no suppressor activity was found in extracts obtained from mice primed with DA (Table XXX). The factor had an apparent molecular weight of between 14,000 and 66,000 daltons and abrogated the anti-DA IgE response of irradiated recipients injected with spleen cells of mice primed with DA (ratio $10^6 : 10^7$) (Figure 21).

The results in this study clearly demonstrated that soluble antigen specifically abrogated the anti-DA reaginic response. In previous studies, the injection of soluble antigen suppressed the IgE response, at best, transiently. However, modified antigens abrogated the IgE response to the antigen. The immune regulation of the IgE response has not been clearly elucidated due to the lack of simplified systems in order to study this question. The preparation of modified antigens has not always been successful, thus the reproducibility of these systems is questionable. Soluble DA is the first "soluble natural antigen" capable of suppressing specifically the IgE response. Thus, studies can be performed to establish if immune response to DA is under Ir gene control, which will lead to further insights and understanding of the cellular and molecular regulation of the IgE response.

CHAPTER 5

GENERAL DISCUSSION

It has been clearly demonstrated that the IgE response to an antigen was abrogated specifically by the administration of modified antigen, OA-MγG or a soluble antigen, DA. The results obtained by these two systems at first, seem contradictory since soluble OA suppressed the IgE response only in a transient fashion and had no effect on the hemagglutinating antibody response (Tables II,V,VI,VII, Figures 8-10) whereas soluble DA suppressed profoundly the anti-DA IgE as well as H.A. antibody responses (Figures 13-16). However, Sercarz et al (181) had observed that certain peptides in a protein molecule regulated the immune response to the antigen. Different peptides would activate different subpopulations of T cells, either helper or suppressor cells, and consequently, the resultant of a variety of determinants of the antigen dictated the magnitude of the immune response to the antigen. Similar to the findings that only certain determinants activated B or T cells, suppressor and helper T cells recognize different determinants of an antigen (181). It is possible that in the case of OA, T helper cells were preferentially stimulated in the mice by the injection of soluble OA, due to the presence of predominatnly Th determinants on the molecule. On the other hand, soluble DA seems to have just the opposite effect since Ts cells were easily activated by the injection of soluble DA.

Furthermore, the presence of adjuvant is critical for the induction of different classes of antibodies especially for IgE antibody production. In unpublished observations, mice injected with low or high doses (0.1,1,10 100 or 200 μg) of OA in saline had no measurable

IgE antibody titers. However, upon challenge with a sensitizing dose of antigen incorporated in $Al(OH)_3$ on day 28, a secondary response was detected, i.e. a higher and earlier response (day 7 vs day 14). These results indicated that soluble antigen induced possibly Th cells and definitely produced memory B_ε cells (i.e. sensitized the animals) since a secondary response was easily achieved upon a secondary challenge with antigen in adjuvant $Al(OH)_3$. The adjuvant stimulated a cell(s) of unknown origin which permitted the expression of the humoral IgE response. It is hypothesized that the cell involved is the macrophage which, when activated, produced factors that stimulated the production of IgE responses. Lang and Filion demonstrated also that the injection of $Al(OH)_3$ into normal nonimmunized rats induced a nonspecific elevation of total IgE level in these animals. These results support the concept that the normal dampening mechanism for the IgE response was circumvented by the injection of $Al(OH)_3$ and the adjuvant stimulated the cellular pathway that is involved in the production of IgE antibodies.

The modification of antigen with MγG had resulted in the formation of tolerogenic conjugates capable of suppressing the IgE response. The conjugation of MγG to OA either may have led to steric hindrance of Th cell antigen determinants, although this is unlikely since T cells recognize primary amino acid structures (168,182) or the conjugates may behave as immune complexes which bind Fc receptors on certain T cells, activating the release of suppressor signals rather than helper signals (161). Moreover, the abrogation of the reaginic antibody response of normal nonirradiated mice by spleen cells of donors administered OA-MγG clearly indicated that suppressor cells were involved

and that the cells probably acted on the efferent arm of the immunological system.

The anti-DA humoral responses (IgE, H.A. and PFC) were abrogated by the injection of soluble DA. In addition, soluble OA or RAG affected only transiently the primary and secondary anti-OA or anti-RAG IgE responses (6,10,11,12,163, 2nd chapter of thesis). In contrast, soluble antigen has been demonstrated by various investigators (reviewed in 170,172,173,178) to dampen the IgG antibody response to the antigen in question. Thus, it seemed that the IgE response was regulated somewhat differently than the IgG response. Ishizaka (182) Lee and Sehon (6) Schwenk et al (105) and Filion (2nd chapter of thesis) have shown clearly that the IgE responses may be specifically abrogated without affecting the IgG response to the antigen in question. Thus, the induction of IgE antibodies in mice seems to be regulated by different mechanism(s). Fox et al (98) demonstrated also that IgE B cells of umprimed or primed mice were much more radio-resistant than primed IgG B cells. Takatsu and Ishizaka (183,184) demonstrated that carrier primed T cells controlled the proportion of B memory cells, committed for different isotypes, and influenced the distribution, of antibodies among different classes. IgE class-specific but antigen-nonspecific Ts cells were also characterized (107,183). Thus, it may be concluded that the IgE antibody response is considerably different as compared to the IgG response, eventhough, in certain experimental conditions, parallel observations between IgE antibody class and the other classes of immunoglobulins have been made.

The suppression of the anti-DA IgE and IgG response by soluble DA can be interpreted to indicate that possibly two mechanisms were involved in the phenomenon. The induction of Ts cells in the termination

of reaginic responses by soluble DA reported in this study is the first clear demonstration that a soluble antigen activated preferably antigen-specific Ts cells. However, Ts cells were not class specific since the IgG response to DA was also affected. Evidence was provided to indicate antigen-specific T-T cell interactions. Ngan and Kind (185) provided also results indicating T-T cell interactions in the regulation of the IgE response. In their system, carrier primed T lymphocytes were shown to be responsible for the inhibitory effects of transferred spleen cells into normal mice. In addition, treating the recipients with mild irradiation or with cyclosphosphamide before cell transfer resulted in the abrogation of suppressor effect of the immune cells. However, the inhibitory effect of the carrier primed cells was re-established by injecting normal spleen cells into the recipients. Furthermore, although the generation of the suppression was antigen specific, the expression of suppression appeared nonspecific.

It may be possible to establish the role of IgG antibodies (if any) in the dampening mechanism of the IgE response. For years, investigators have laboured over this question but failed to determine unequivocally the role of IgG antibodies in the suppression of IgE antibody production. The cellular mechanism involved in the long lasting suppression induced by OA-MYG and soluble DA may involve more than one mechanism. The dampening effect of the suppressive agents in question was studied relatively early in the immune response. Kinetic measurements of the activity of the different cell types in both systems described herein must be performed in order to determine the cellular mechanism(s) involved in the long term dampening of the reaginic response. It is possible that memory Ts cell are reactivated

with subsequent immunizations as demonstrated by Schwenk et al (105). However, other mechanism such as an idiotypic network control mechanism may also be involved in the long term regulation of responses to antigen (175,105). Further experimentation to answer these questions is needed.

Furthermore, a soluble suppressive factor was extracted from the Ts cells induced by soluble DA and found to have a similar molecular weight as the suppressor factor characterized by Tada (180), and Kapp et al (59). The characterization of the suppressive factor, such as heat stability, susceptibility to various enzymes, antigen specificity, target cell, genetic characteristics, etc, was not undertaken in this study, since it represents another major research project. Moreover, these results will lead to a clearer understanding of the regulatory mechanism controlling the reaginic response.

CONCLUSION

The induction of suppressor cells was achieved by the administration of modified antigen (OA-MγG) or soluble antigen (DA) to mice. Soluble OA suppressed transiently the IgE response and thus, it was concluded that not all soluble forms of antigens suppressed the IgE response effectively. However, these antigens when properly modified by conjugation to nonimmunogenic carriers, may specifically abrogate the production of IgE antibodies. Thus, the administration of OA-MγG generated suppressor cells which had the ability of depressing the potential of both normal and primed animals to mount reaginic antibody responses to ovalbumin and to a hapten attached to it. This evidence also strongly suggests that Th cells are probably the target cells in the overall mechanism responsible for suppression. Experiments must be performed in order to identify the nature of the suppressor cells and their target cells, since, in principle, it may be visualized that suppressor cells may act either on Th cells or on the appropriate B cells or on both.

Moreover, not all antigens need modification in order to become a suppressive agent for the IgE response. The induction of an unresponsive state with the aid of soluble DA was easily achieved in normal mice or mice primed with DA. The Ts cells induced were antigen specific and by the use of an adoptive transfer system, it was concluded that the Ts cells were effector cells and not inducer Ts cells since, these cells acted preferentially in irradiated host and were incapable of dampening the reaginic response of mice primed with DA. Nevertheless, it is possible that a population of inducer Ts cells does exist also in these animals. Further experimentation is needed before a final conclusion is drawn.

It was concluded also that the target cell for Ts cell was another T cell probably Th cell, since it was clearly demonstrated that helper function was diminished in mice treated with soluble DA. However, it is unknown if the Th cells were paralyzed or eliminated in treated mice. The population of B cells was unaffected by the treatments since B cells from suppressed mice were able to cooperate with Th cells from mice primed with DA in an adoptive cell transfer system. Thus, it was concluded that soluble DA induced suppressor cells mainly affecting Th cells. Moreover, a soluble suppressor factor was extracted from either whole spleen or enriched splenic T cells of mice treated with soluble DA and found to have a similar molecular weight as factors described by Tada (180) and Kapp et al (59). Furthermore, the cellular mechanism maintaining the unresponsive state had not been elucidated and may involve a variety of different cellular pathways. Further experiments must be performed in order to clearly understand the cellular mechanism(s) involved in this experimental system.

From the results of the two experimental systems described in this thesis, it may be concluded that soluble antigen may or may not affect IgE response which seems to depend on the nature of the antigen. However, antigen processing by macrophages or the lack of processing may be involved in the induction of unresponsive states (167,168) Sercarz et al (181) has demonstrated that Ts cells recognize different antigenic determinants than B cells and Th cells. Thus, the presence or absence of certain determinants will determine if Ts will be activated. Therefore, it was concluded that soluble DA must have at least one such determinant which is sufficient to activate Ts cells. In contrast, OA

does not possess any such determinants or that the affinity of Ts receptors for determinants on OA are very weak and these cells are unable to be activated by such a weak interaction. However, the conjugation of OA onto nonimmunogenic carriers leads to a stabilization of the Ts cell receptor with Ts cell antigenic determinants on OA which leads to the activation of such cells.

REFERENCES

1. Katz, D.H.
in "Immunological Tolerance: Mechanisms and Potential Therapeutic Applications " (eds Katz, D.H. and Benacerraf B.), Academic Press, N.Y., N.Y. p. 89, 1974.

2. Bach, M., and Brashler, J.
J. Immunol. 114, 1799, 1975.

3. Lee, W.Y. and Sehon, A.H.
J. Immunol. 114, 829, 1975.

4. Lee, W.Y. and Sehon, A.H.
J. Immunol. 114, 837, 1975.

5. Takatsu, K., and Ishizaka, K.
Fed. Proc. 34, 1000 (Abs 4425).

6. Lee, W.Y. and Sehon, A.H.
Immunol. Rev. 41, 200, 1978.

7. Marsh, D.G.
Int. Arch. Allergy appl. Immunol. 41, 119, 1976.

8. Moran, D.M., and Wheeler,A.W.
Int. Arch. Allergy appl. Immunol. 50, 693, 1976.

9. Moran, D.M., and Wheeler, A.W.
Int. Arch. Allergy appl. Immunol. 50, 699, 1976.

10. Liu, F.T., and Katz, D.H.
Proc. Nat. Acad. Sci. (USA) 76, 1430, 1979.

11. Liu, F.T., Bogowitz, C.A., Bargatze, R.F., Zinnecker, M., Katz, L.R., and Katz, D.H.
J. Immunol. 123, 2456, 1979.

12. Usui, M., and Matuhasi, T.,
J. Immunol. 122, 1266, 1979.

13. Humphrey, J.H., Parrot,D.M.V., and East, J.,
Immunology

14. Claman, H.N., Chaperon, E.A., and Triplet, R.F.
Proc. Soc. Exp. Biol. Med. 122, 1167, 1966.

15. Kennedy, S.C., Siminovitch, L., Till, J.E. and McCulloch, E.A.
Proc. Soc. Exp. Biol, Med. 120, 868, 1965.

16. Playfair, J.H., Papermaster, B.W., and Cole, L.J.
Science, 149, 998, 1965.

17. Miller, J.F.A.P., and Mitchell, G.F.
Nature, 21, 659, 1967

18. Mitchell, C.F., and Miller, J.F.A.P.
Proc. Nat. Acad. Sci. (USA) 59, 296, 1978

19. Dutton, R.W.
Adv. Immun. 6, 254, 1967

20. Mishell, R.I., Dutton, R.W.
J. Exp. Med. 126, 423, 1967

21. Marbrook, J.
Lancet, 2,1279, 1967

22. Mosier, D.E., and Coppleson, L.W.
Proc. Nat. Acad. Sci. (USA) 61, 542, 1968

23. Mosier, D.E., Fitch, F.W., Rowley, D.A. and Davies, A.J.S.
Nature, 225, 276, 1970

24. Chiller, J.M., Habicht, G.S. and Weigle, W O.
Proc. Nat. Acad. Sci. (USA) 65, 551, 1970

25. Miller, J.F.A.P., and Sprent, J.
J. Expt. Med. 134, 66, 1971

26. Tittor, W., and Walford, R.L.
Nature, 247, 371, 1974

27. Landsteiner, K.
1962. "The specificity of Serological Reactions (rev. ed.)
Dover, N.Y.

28. Benacerraf, B., Ojeda, A., and Maurer, P.H.
J. Exp. Med. 118, 945, 1963

29. Green, I., Paul, W.E. and Benacerraf, B.
J. Exp. Med. 123, 859, 1966

30. Levine, B.B., Ojeda, A., and Benacerraf, B.
Nature, 200, 544, 1963

31. Ovary, Z., and Benacerraf, B.
Proc. Soc. Exp. Biol. Med. 14, 72, 1963

32. Mitchison, N.A.
in "Immunological Tolerance" M. Ladez and N. Brawn eds
p. 149 Academic Press, N.Y.

33. Raff, M.C.
Nature, 226, 1257, 1970

34. Niederhuber, J.E., Moller, E., and Makela, O.
Eur. J. Immunol. 2, 371, 1972

35. Rajewsky, K., Schirrmacher, V., Nase, S. and Jerne, N.K.
J. Exp. Med. 129, 1131, 1969

36. Katz, D.H., Paul, W.E., Goidl, E.A., and Benacerraf, B.
J. Exp. Med. 132, 261, 1970

37. Paul, W.E., Katz, D.H., Goidl, E.A. and Benacerraf, B.
J. Exp. Med. 132, 283, 1970

38. Gershon, R.K.
Contemp. Topics Immunobiol. 3, 1, 1973

39. Liacopoulos, P., and Ben-Efraim, S.
Prog. Allergy, 18 97, 1975

40. Pierce, C.W., and Kapp, J.A.
Contemp. Top. Immunobiol. 5, 91, 1976

41. Katz, D.H.
"Lymphocyte differentiation, Recognition and Regulation"
Academic Press N.Y., N.Y. 1977

42. Gershon, R.H. and Kondo, K.
Immunol. 18, 723, 1970

43. Gershon, R.H. and Hensin, R.S.
J. Immunol. 107, 1723, 1971

44. Katz, D.H., Paul, W.E., and Benacerraf, B.
J. Immunol. 110, 107, 1972

45. Tada, T.
in Immunological Tolerance: Mechanisms and Potential Therapeutic Applications. (D.H. Katz and B. Benacerraf eds) p. 471 Academic Pres. N.Y., N.Y.

46. Tada, T., Okumura, K., and Taniguchi, M.
J. Immunol. 111, 952 1973.

47 Okumura, K., and Tada, T.
J. Immunol. 112, 783, 1974

48. Takimori, T., and Tada, T.
J. Exp. Med. 140, 253, 1974

49. Takimori, T. and Tada, T.
J. Exp. Med. 142, 1241, 1975

50. Taniguchi, M., Hayakawa, K., and Tada, T.
J. Immunol. 116, 542, 1976

51. Taniguchi, M., Tada, T., and Tokuhisa, T.
J. Exp. Med. 144, 20, 1976

52. Tada, T. Taniguchi, M. and David, C.S.
Cold Spring Harbour, Symp. quant. Biol. 41, 1977

53. Tada, T., Takemori, T., Tokuhisa, T., Taniguchi, M., and Okumura, K.,
International congress of Allergology, 1976 p. 95

54. Gershon, R.K., Maurer, P.H., and Merryman, C.F.
Proc. Nat. Acad. Sci. (USA) 70, 250, 1973

55. Kapp, J.A., Pierce, C.W., and Benacerraf, B.
J. Exp. Med. 138, 1107, 1973

56. Kapp, J.A., Pierce, C.W., and Benacerraf, B.
J. Exp. Med. 138, 1121, 1973

57. Kapp, J.A., Pierce, C.W., and Benacerraf, B.
J. Exp. Med. 140, 172, 1974

58. Pierce, C.W., Kapp, J.A., and Benacerraf, B.
J. Exp. Med. 144, 371, 1976

59. Kapp, J.A., Pierce, C.W., and Benacerraf, B.
1976 in "The Role of Products of the Histocompatibility Gene Complex in Immune Responses." D.H. Katz and B. Benacerraf eds). p. 569 Academic Press, N.Y.

60. Benacerraf, B., and Germain, R.N.,
Immunol. Rev. 38, 10, 1978

61. Benacerraf, B., and Germain, R.N.
Fed. Proc. 38, 2053, 1979

62. Eardley, D.D., Hugenberger, J.L., McVay-Bourdreau, L., Shen, F.W., Gershon, R.K., and Cantor, H.
J. Exp. Med. 147, 106, 1978

63. Gershon, R.K., Cohen, P., Hensin, R.S., and Liebhaber, S.A.,
J. Immuno. 108, 586, 1972

64. Block, K.J.
Prog. Allergy 10, 84, 1967

65. Block, K.S.,
In " Mechanism in Allergy" p. 11 Dekker, N.Y., 1975

66. Ishizaka, K.
John Hopkins Med. J. 135, 67, 1975

67. Prouvost-Danon, A., Silva Lima, M., and Queiroz Javierre, M.,
Life Science 5, 289, 1966

68. Mota, I.,
Immunol, 7, 681, 1964

69. Binaghi, R.A., and Benacerraf, B.,
J. Immnol. 92, 920, 1964

70. Ogilvie, B.M.,
Immunol. 12, 13 1967

71. Ogilvie, B.M., and Jones, V.E.,
Exp. Parasitol. 29, 138, 1971

72. Levine, B.B., and Vaz, N.M.,
Int. Arch. Allergy Appl. Immunol. 39, 156, 1970

73. Vaz, E.M., Vaz, N.M., and Levine, B.B.
Immunology 21, 11, 1971

74. Revoltella, R., and Ovary, Z.
Immunol. 17, 45, 1969

75. Dorf., M.E., Newburger, P.E., Hamoaka, T., Katz, D.H., and Benacerraf B.
Eur. J. Immunol. 4, 346, 1974

76. Dessein, A., Germain, R.N., Dorf, M.E. and Benacerraf, B.
J. Immunol. 123, 463, 1977

77. Levine, B.B., Stimber, R.H., and Fotino, M.,
Science, 178, 1201, 1972

78. Marsh, D.G., Bias, W.B., Hsu, S.H. and Goodfriend, L.,
Science, 179, 691, 1973

79. Stannegard, O., and Yurchison, A.,
Immunol. 16, 387, 1969

80. Strejan, G.H., and Marsh, D.G.
J. Immunol, 107, 306, 1971

81. Tada, T., and Okumura, K.
J. Immunol. 107, 1137, 1971

82. Hamaoka, T., Katz, D.H., and Benacerraf, B.,
J. Exp. Med. 138, 538, 1973

83. Ishizaka and Kishimoto, T. in
"Control Mechanism in Reagin-Mediated Hypersensitivity"
(L. Goodfriend ed.) p. 51 Dekker, N.Y., 1976

84. Tada, T. Okumura, K., and Taniguchi, M.
J. Immunol. 108, 1535, 1972

85. Okudaira, H., and Ishizaka, K.,
J. Immunol, 111, 1420, 1973

86. Okumura, K., and Tada, T.,
J. Immunol. 106, 1019, 1971

87. Taniguchi, M., and Tada, T.,
J. Immunol. 113, 1757, 1974

88. Hamaoka, T., Katz, D.H., Bloch, K.J. and Benacerraf, B.
J. Exp. Med. 138, 306, 1973

89. Micheal, J.G., and Bernstein, I.L.,
J. Immunol. 111, 1600, 1973

90. Tada, T.,
in: Immune Recognition
A.S. Rosenthal, ed. p. 771 Academic Press
N.Y. 1975

91. Tada, T., Taniguchi, M., and Okumura, K.,
J. Immunol. 106, 1012, 1971

92. Taniguchi, M., and Tada, T.,
J. Immunol. 107, 579, 1971

93. Zvaifler, N.J. and Robinson, J.O.,
J. Immunol. 102, 947, 1969

94. Okumura, K., and Tada, T.,
J. Immunol. 107, 1682, 1971

95. Kind, L.S. and Ako, D.
Transpl. 11 489, 1971

96. Kind, L.S., and Macedo-Sabrinho, B.,
Transpl. 14 317, 1972

97. Kind, L.S., and Macedo-Sabrinho, B.,
Int. Arch. Allergy, Appl. Immunol. 45 780, 1973

98. Fox, D., Chiorazzi, N., and Katz, D.H.,
J. Immunol. 117, 1622, 1977

99. Chiorazzi, N., Fox, D., and Katz, D.H.
J. Immunol. 117, 1629, 1976

100. Watanabe, N., Kojima, and Ovary, Z.,
J. Exp. Med. 143, 833, 1976

101. Wataneba, N., and Ovary, Z.,
J. Immunol. 118, 485, 1977

102. Feldman, M., Beverley, P.C.L., Dunkley, M., Kontiainen, S.,
Nature, 258, 614, 1975

103. Cantor, H., Shen, F.W. and Boyse, E.A.J.
Exp. Med. 143, 1391, 1976

104. Tamura, S., and Ishizaka, K.,
J. Immunol. 120, 837, 1978

105. Schwenk, R.J., Kudo. K., Miyamoto, M., and Sehon, A.H.
J. Immunol. 123, 2791, 1979

106. Gollapudi, V.S.S. and Kind, L.S.
J. Immunol. 114, 906, 1975

107. Kishimoto, T., Hirai, Y., Suemura, M., and Yamamura, Y.,
J. Immunol. 117, 396, 1976

108. Kimoto, M., Kishimoto, T., Noguchi, S., Watanabe, F., and Yamamura, Y.,
J. Immunol, 118, 840, 1977

109. Suemura, M., Kishimoto, T., Hirai, Y., and Yamamura, Y.,
J. Immunol. 119, 149, 1977

110. Kishimoto, T., Hirai, Y., Suemura, M., Nakanishi, K., and Yamamura, Y.,
J. Immunol. 121, 2106, 1978

111. Tung, A.S., Chiorazzi, N., and Katz, D.H.,
J. Immunol. 120, 2050, 1978

112. Katz, D.H., and Tung, A.S.
J. Immunol. 120, 2060, 1978

113. Katz, D.H., Bargatze, R.F. Begowitz, C.A., and Katz, L.R.
J. Immunol. 122, 2184, 1979

114. Katz, D.H., Bargatze, R.F., Bogowitz, C.A., and Katz, L.R.
J. Immunol. 122, 2191 1979

115. Havas, H.F.,
Immunol. 17, 819, 1969

116. Golan, D.T., and Borel, Y.,
J. Exp. Med, 134, 1046, 1971

117. Hamilton, J.A., and Miller, J.F.A.P.,
Eur. J. Immunol. 4, 261, 1974

118. Hamilton, J.A., Miller, J.F.A.P., and Kettman, J.,
Eur. J. Immunol. 4, 268, 1974

119. Katz, D.H., Davie, J.M., Paul, W.E., and Benacerraf, B.,
J. Exp. Med. 134, 201, 1971

120. Katz, D.H., Hamaoka, T., and Benacerraf, B.,
J. Exp. Med, 136, 1404, 1972

121. Katz, D.H., Hamaoka, T., and Benacerraf, B.
Proc. Nat. Acad. Sci. (USA) 70, 2776, 1973

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

123. Lee, W.Y., and Sehon, A.H.,
J. Immunol. 117, 927, 1976

124. Lee, W.Y., and Sehon, A.H.,
J. Immunol. 116, 1711, 1976

125. Desaymard, C.,
Eur. J. Immunol. 7, 646, 1977

126. Watanabe, N., Kojima, S., and Ovary, Z.,
J. Immunol. 118, 251, 1977

127. Diner, U.E., Kinimoto, D., and Deiner, E.,
J. Immunol. 122, 1886, 1979

128. Lee, W.Y. and Sehon, A.H.,
Immunological Letters, 1, 31, 1979

129. Hubbard, D.,
in "Suppression of reaginic antibody production with polyvinyl alcohol as tolerogenic carrier." M.Sc. Thesis, University of Manitoba, 1979

130. Lee, W.Y., Hubbard, D.A. and Sehon, A.H.,
submitted to J. Immunol, 1979

131. Takatsu, K., and Ishizaka, K.,
Cell. Immunol. 20, 276, 1975

132. Takatsu, K., and Ishizaka, K.,
J. Immunol. 116, 1257, 1976

133. Takatsu, K., and Ishizaka, K.,
J. Immunol. 118, 151, 1977

134. Lee, W.Y., and Sehon, A.H.,
Int. Arch. Allergy Appl. Immunol. 56, 159, 1978

135. Lee, W.Y., and Sehon, A.H.
Int. Arch. Allergy Appl. Immunol. 56, 193, 1978

136. David, M.F.,
J. Allergy, Clin. Immunol. 60, 180, 1977

137. Vaz, N.M., Maia, L.C.S., Hornbrook, M.M., Lynch, J.M., and Roy, C.A.
J. Allergy Clin. Immun. 60, 110, 1977

138. Ngan, J., and Kind, L.S.
J. Immunol. 120, 861, 1978

139. Mattingly, J.A. and Waksman, B.H.,
J. Immunol. 121, 1878, 1978

140. Hanson,D.G., Vaz, N.M., Rawlings, L.A., Lynch, J.M.,
J. Immunol. 122, 2261, 1979

141. Hrabra, T., Havas, H.F., and Pickford, A.R.,
Int. Arch. Allergy Appl. Immunol. 38, 635, 1970

142. Borel, Y.,
Immunol. Rev. 31, 1, 1976

143. Miller, S.D., and Claman, H.N.,
J. Immunol. 117, 1519, 1978

144. Nossal, G.J.V., Pike, B.L., and Katz, D.H.,
J. Exp. Med. 138, 312, 1973

145. Mitchell, G.F., Humphrey, J.H., and Williamson, A.R.,
Eur. J. Immunol. 2, 460, 1972

146. Katz, D.H.
in "Immunological Tolerance: Mechanisms and Potential Therapeutic Applications." (eds. Katz, D.H., and Benacerraf, B.,) Academic Press. N.Y., N.Y. p. 249, 1974

147. Pan, D., Lee, W.Y., Bazin, H., and Sehon, A.H.,
Fed. Proc. 35, 433, 1976

148. Lee, W.Y., Kepron, W., Sehon, A.H., and Tse, K.S.,
European Immunology Meeting, Amsterdam, Sept. 1975, Abstract.

149. Berrens, L., Henocq, E., and Rudermecker, M.,
Clin. Allergy, 3, 449, 1973

150. Avrameas, S.,
Immunochem. 6, 43, 1969

151. Ouchterlony, C.,
Progress in Allergy 5, 1, 1958

152. McConachy, P.J., and Dixon, F.,
Int. Ach. Allergy Apl. Immunol. 29, 1985, 1966

153. Williams, C.A. and Chase, M.W.

"Methods in Immunology and Immunochemistry", 2, 343, 1968

154. Janeway, C.A., and Humphrey, H.,
Immunol. 14, 225, 1968

155. Schultze, H.E.,
Molecular Biology of Human Proteins
Vol 1. Elsevier Publishin Co. Amsterdam.

156. Weigle W.O.,
Adv. Immunol. 16, 61, 1973

157. Pierce, C.W., Kapp, J.A., and Benacerraf, B.,
Cold Spring Harbour Symp. 41, 563, 1977

158. Erb. P., and Feldman, M.J.
J. Exp. Med. 142, 460, 1975

159. Skidmore, B.J., and Katz, D.H.,
J. Immunol. 119, 694, 1977

160. Hodes, R., Ahmann, G., Hathcock, K., Dickler, H.,
and Singer, A.
J. Immunol. 121, 1501, 1978

161. Basten, A., Miller, J.F.A.P., and Johnson, P.,
Transpl. Rev. 26, 130, 1974

162. Jerne, N.K., Nordin, A.A., and Henry, C.,
in " Cell-bound Antigen" p. 109
Philadelphia, Western Institute Press.

163. Katz, D.H.,
Immunol. Rev. 41, 77, 1978

164. Cunningham, A.J., and Sercarz, E.E.,
Eur. J. Immunol. 1, 413, 1971

165. Hamaoka, T., Newburger, P.E., Katz D.H., and Benacerraf, B.
J. Immunol. 113, 958, 1974

166. Chiller, J.M., and Weigle, N.O.,
Transpl. Rev. 8, 3, 1971

167. Benacerraf, B., and Germain, R.N.
Immunol. Rev. 38, 70, 1978

168. Rosenthal, A.S.
Immunol. Rev. 40, 136, 1978

169. Parish, C., Kirov, S., and Bowern, M., and Blanden, R.,
Eur. I. Immunol. 4, 808, 1974

170. Gershon, R.K.
Transpl. Rev. 26, 180, 1975

171. Rajewsky, K., and Brenig, C.,
Eur. J.Immunol. 4, 120, 1974

172. Weigle, W.O. Sieckman, D.G., Doyle, M.V., and Chiller, J.M.,
Transpl. Rev. 26, 186, 1975

173. Nachtigal, D., Zan-Bar, I., and Feldman, M.,
Transpl. Rev. 26, 85, 1975

174. Miller, S.D., Man-Sun, S., and Claman, H.N.,
J. Immunol. 121, 265, 1978

175. Zan-Bar, I., Murphy, D.B., and Strober, S.
J. Immunol. 120, 497, 1978

176. Cantor, H., McVay-Bourdreau, L., Hugenberger, J., Naidorf, K., Shen, F.W., and Gershon, R.K.
J. Exp. Med, 147, 1116, 1978

177. Jerne, N.K.,
Ann. Immunol. Int. Pasteur, 125C 373, 1974

178. Kapp, J.A., Pierce, C.W., Schlossman, S., and Benacerraf. B.,
J. Exp. Med. 140, 648, 1974

179. Segal, S., Tzehoval, E., and Feldman, M.,
Proc. Nat. Acad. Sci. (USA) 76, 2405, 1979

180. Tada, T.,
Progress in Allergy, 19, 122, 1975

181. Sercarz, E.E., Yowell, R.L., Turkin, D., Miller, A., Araneo, B.A, and Adorini, L.,
Immunol. Rev. 39, 108, 1978

182. Benacerraf, B.,
J. Immunol, 120, 1809, 1978

183. Ishizaka, K., and Ishizaka, T.,
Immunol. Rev. 41, 107, 1978

184. Takatsu, T., and Ishizaka, K.,
J. Immunol. 114, 1938, 1975

185. Ngan J., and Kind, L.S.,
Int. Arch, Allergy, Appl Immunol. 58, 195, 1979