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Modulation Of The Immunoglobulin-e Response By Dnp-coupled Bordetella Pertussis

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MODULATION OF THE IgE RESPONSE BY DNP-COUPLED *BORDETELLA PERTUSSIS*

by

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Submitted in partial fulfillment

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"If we really knew everything we should know, we could be discouraged, depressed and intellectually sterilized, the scientist often avoids being learned because erudition kills imagination"

Andre Lwoff

ABSTRACT

Immunological adjuvants potentiate both the cell-mediated and antibody-mediated arcs of the immune response in an antigen-nonspecific manner. Adjuvants are known, however, to modulate selectively the immune response of various immunoglobulin classes. Thus complete Freund's adjuvant (CFA) augments cell-mediated immune responses and IgG antibody synthesis while at the same time dampens antibody responses of the IgE class. Bordetella pertussis vaccine and aluminum hydroxide gel (alum) preferentially potentiate IgE antibody responses.

The purpose of the present investigation was to examine the IgE-enhancing effects of B. pertussis under conditions in which a hapten (2,4-dinitrophenyl) was covalently attached to their cell wall. It was expected that the administration of DNP-B. pertussis (DNP-BP) into mice would lead to an increased production of anti-DNP antibodies of the IgE class and efforts could then be made to modulate this response at will.

Contrary to expectations, administration of DNP-BP to CBA mice resulted in negligible anti-DNP IgE production following either primary or secondary stimulation, even when DNP-BP was administered in association with an additional adjuvant (alum). The inability to stimulate IgE antibodies was not due to an intrinsic defect of DNP-BP since anti-DNP IgG antibodies were exceptionally high and the administration of DNP-BP to a number of inbred mouse strains differing in H-2 genotype showed a clear segregation into low and high IgE responder phenotypes. Furthermore, immunization of CBA mice with DNP-OA in alum 2 weeks following administration of DNP-BP

resulted in an accelerated anti-DNP IgE response (day 7) followed by a drastic (more then 90%) decrease of IgE but not of IgG anti-DNP levels by day 14-21. The results indicated that treatment with DNP-BP is capable of priming IgE B-memory cells but that a powerful IgE-selective suppressive mechanism follows, preventing the development of normal IgE antibody levels.

The failure of CBA mice to mount the expected IgE anti-DNP responses following treatment with DNP-BP and immunization with DNP-OA in alum, provided the opportunity to explore the mechanisms involved in this suppression.

The suppression induced by treatment with DNP-BP was insensitive to treatment of mice with cyclophosphamide or with low dose x-irradiation. The adoptive transfer of spleen cells from DNP-BP-treated donors to normal syngeneic recipients resulted in a weak and delayed (day 21) but statistically significant suppression of the anti-DNP IgE antibody response, following priming of the recipients with DNP-OA alum. Depletion of T cells from the transferred population did not modify this suppressive pattern. Both in situ and in adoptive recipients, the suppression was DNP-specific. The results suggested that IgE-selective, hapten (DNP)-specific suppression operates through T-independent mechanisms, although the induction of these mechanisms may rely on an intact functional thymus.

Passive administration of the serum obtained from DNP-BP-treated, DNP-OA immunized mice (following depletion of anti-DNP and anti-OA antibodies) did suppress the induction as well as an ongoing anti-DNP IgE antibody response induced against DNP-OA alum in syngeneic recipients.

Furthermore, the treatment of such serum with anti-DNP antibodies inhibited the binding of iodinated-DNP-bovine serum albumin (DNP-BSA) in a radioimmunoassay inhibition test, demonstrating the anti-idiotypic activity. The anti-idiotypic activity was found associated with a serum fraction eluting with the bulk of immunoglobulins, from sephadex G-100.

Results strongly suggested the possibility that the IgE-selective, hapten-specific suppression in CBA mice operates via auto-anti-idiotypic antibodies directed against one or more DNP-specific predominant idiotypes.

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LIST OF ABBREVIATIONS

aId	Anti-idiotype
alum	$Al(OH)_3$
AS	absorbed serum
ASC-1	purified Ascaris allergen
BSA	bovine serum albumin
BP	Bordetella pertussis
BSS	balance salt solution
BPO	benzylpenicilloyl
CE	Ascaris crude extract
CFA	complete Freund's adjuvant
Con-A	concanavalin A
CRIA	cross-reactive idiotype A
CY	cyclophosphamide
DEAE	diethylaminoethyl
DNP	dinitrophenyl
DNP-BP	Bordetella pertussis organisms modified with dinitrophenyl hapten
DNP-CE	Ascaris crude extract modified with dinitrophenyl hapten
DNP-MYC	Mycobacterium tuberculosis modified with dinitrophenyl hapten
DNP-PC	Phosphorylcholine modified with dinitrophenyl hapten
FCS	fetal calf serum
Fr	fraction

GAT	L-glutamic acid^{60}-L-alanine^{60}-L-tyrosine^{10}
IFA	Incomplete Freund's adjuvant
Ig	immunoglobulin
i.d.	intradermal
i.m.	intramscular
i.p.	intraperitoneal
i.v.	intravenous
KLH	Keyhole limpet hemocyanin
LPS	lipopolysaccharide
NAS	nonabsorbed serum
NMS	normal mouse serum
OA	ovalbumin
PBS	phosphate buffered saline
PC	phosphorylcholine
PCA	passive cutaneous anaphylaxis
PFC	plaque forming cell
RAMG	rabbit-anti mouse IgG
RaMIg	rabbit-anti mouse immunoglobulins
RIA	radioimmunoassay
TBET	trypan blue exclusion test
TNP	trinitrophenyl
TNP-Ficoll	Ficoll modified with trinitrophenyl hapten
TNP-KLH	Keyole limpet hemocyanin modified with trinitrophenyl hapten
Tris	tris(hydroxymethyl)aminomethane

1. INTRODUCTION

The IgE antibody system has great importance because of its well-established role in the pathogenesis of human allergic diseases. Therefore, specific suppression of the IgE response in an antigen-specific manner is therapeutically desirable.

Recent investigations in several laboratories have revealed that the IgE antibody response operates under the control of unique regulatory T cells acting in a complex but highly coordinated fashion (Ishizaka, 1980; Katz, 1981). In experimental animals the production of IgE antibodies reaches detectable serum levels only if the immunogen is administered with certain adjuvants. Among these Bordetella pertussis (BP) vaccine and aluminum hydroxide gel (alum) are very effective while incomplete, and complete Freund's adjuvant (IFA and CFA) are not (Revoltella and Ovary, 1969; Tada, 1975; Katz, 1978b). The failure of CFA to promote IgE antibody production appears to be associated with its property to stimulate the production of T cell derived regulatory molecules that suppress selectively the IgE antibody response in an antigen-nonspecific fashion (Kishimoto et al., 1978; Katz, 1978a; Katz et al., 1978; Hirashima et al., 1982).

BP vaccine acts as an adjuvant with most soluble antigens in many animal species to enhance IgE antibody production more effectively than other adjuvants (Mota 1964; Clausen et al., 1969). The adjuvant effect of BP vaccine has been attributed to a component called leukocytosis-promoting factor (LPF) (Morse and Morse, 1976) or "Pertussigen" (Munoz and Bergman, 1977) a protein molecule associated with the cell wall of smooth strains, but isolated usually from supernatants of older cultures (Munoz et al, 1959; Morse and Morse,

1976). Various mechanisms were proposed to define the adjuvant effect of BP vaccine but the actual detailed mechanism of its action remained obscure (Munoz and Bergman, 1977). Hirashima et al., (1981a, 1981b) showed that in Lewis rats, the administration of BP vaccine leads to the stimulation of several lymphokines which act synergistically to promote the production of IgE binding factors displaying IgE-potentiating properties. In a recent study done by Iwata et al., (1983), the role of "Pertussigen" was defined in IgE production. They showed that Pertussigen stimulated the production of glycosylation-enhancing factor by spleen cells of Lewis rats.

Conversely, administration of CFA leads to the production of IgE binding molecules with suppressive activity.(Hirashima et al., 1981c, 1982). In the final analysis the main difference between the IgE-binding molecules with enhancing and suppressive activities could be explained by the presence or absence of a mannose-rich carbohydrate, the synthesis of which was found to be contingent upon the relative concentration of glycosylation-enchaning and glycosylation-inhibiting lymphokines (Yodoi et al., 1980; Hirashima et al., 1981, 1982). In a separate series of investigations Kishimoto et al., (Kishimoto et al., 1976, 1978; Suemura et al., 1977; Sugimura et al., 1982) showed that mice injected with dinitrophenyl (DNP)- or phorsphorylcholine (PC)- coupled Mycobacterium tuberculosis produced IgE-selective T-suppressor cells and suppressive molecules. The suppressive effect was antigen-nonspecific but required hapten-specific restimulation of the T cells. The present investigation was initiated with the hope that administration of dinitrophenylated-B. pertussis (DNP-BP) as a

conjugate would induce high levels of anti-DNP IgE antibodies in mice. Efforts could then be made to therapeutically regulate the IgE response in an antigen-specific manner.

The results will show that contrary to our expectation CBA mice immunized with DNP-BP in alum produced no or negligible anti-hapten IgE antibody responses. The failure to mount an IgE response was not attributable to an intrinsic defect of the antigen (DNP-BP) since, excellent levels of IgG antibodies were consistently detected; moreover, a clear pattern of good and poor responsiveness with respect to IgE antibodies was observed when several inbred strains of mice were immunized with this conjugate. Pretreatment of CBA mice with DNP-BP resulted in suppressed anti-DNP IgE but not IgG responses following conventional immunization with DNP-ovalbumin (DNP-OA) in alum. However, the suppression was hapten-specific, apparently independent of T cells, appeared relatively late and followed the appearance in the serum of auto-anti-idiotypic activity.

2. HISTORICAL REVIEW

In 1921 Prausnitz and Kustner first demonstrated that anaphylactic sensitivity could be transferred locally by the serum from a sensitive patient into the skin of a non-sensitive individual. Following careful studies that associated IgE with antibody-mediated skin sensitizing activity (Ishizaka et al., 1966a, 1966b; Ishizaka and Ishizaka, 1967), a variety of clinical manifestations were shown to be mediated by this immunoglobulin (Ig) class (Butcher et al., 1976; Maccia et al., 1976; Chan-Yeung et al., 1978). These include extrinsic asthma, hay fever, rhinitis, urticaria and many drug and occupational allergies. The discovery of IgE antibody as a carrier of reaginic activity opened new avenues for immunologists, to understand the mechanisms involved in its regulation and to find out the means to control its production.

Most of the basic information about IgE antibodies became available from the studies done with sera from atopic patients. Johansson and Bennich (1967) accidently discovered an atypical (myeloma) Ig from a patient, subsequently identified as gamma E globulin (Bennich et al., 1969), and provided an opportunity to study it in detail.

2.1 Animal Models For The Study of The IgE Responses:

Mota (1964) and Binaghi et al., (1964), first demonstrated the production of reaginic antibodies in rats by immunization with antigen in association with B. pertussis vaccine. Though the IgE antibody was also induced in various other experimental animals by several investigators (Zvaifler et al., 1966; Rockey et al., 1967;

Mota, 1969; Weiszer et al., 1968; Revoltella and Ovary, 1969; Clausen et al., 1969; Levine et al., 1971; and Hammer et al., 1971), the earlier investigations had proved that generally the patterns of antibody production obtained, differed strikingly from that seen in spontaneous human atopic diseases. Revoltella and Ovary (1969) did a comparative study on IgE antibody production in different mouse strains and found that mice immunized under identical conditions responded differently (low and high IgE responder mice).

Concomitantly, it became possible to elicit a persistant and bobsterable IgE response in high responder strains of mice employing suitably spaced, repeated minute doses of potent immunogens together with an appropriate adjuvant (Levine and Vaz, 1970). Some of the fundamental knowledge regarding IgE regulation was obtained from the studies done in the mouse and rat. Among these two, however, the mouse system has provided the best experimental model to study the regulation of the IgE response (Ishizaka, 1982). Occurence of high and low IgE responder strains of mice (Revoltella and Ovary, 1969) ideally represent atopic and non-atopic human populations. It was thus possible to study the basic mechanisms involved in IgE reponse and the subsequent studies were directed towards the search of immunological means to prevent and suppress the IgE induction selectively.

It has gradually been established that the route of injection, dose and physical state of the antigen, the adjuvant employed and the animal strain under study, are some of the very important factors which influence IgE antibody production (Clausen et al., 1969, 1970; Revoltella and Ovary, 1969; Levine and Vaz, 1970; Vaz et al., 1971).

Adjuvants play a crucial role in the stimulation and modulation of the IgE antibody response in experimental animals. Immunization of rodents with soluble antigens without adjuvant failed to elicit IgE responses (reviewed by Ishizaka, 1976). However, more recent studies (Taylor et al., 1980; Holt et al., 1981; Kudo et al., 1981) have documented the induction of persistent and boosterable IgE response in inbred mice, employing soluble protein antigens administered without adjuvant. B. pertussis vaccine (Mota and Peixoto, 1966; Suko et al., 1977) and B. pertussis extracts (Clausen et al., 1969, 1970; Tada et al., 1972; Lehrer et al., 1975; Munoz and Bergman, 1977; Mizushima et al., 1979) were used as adjuvant to potentiate the reaginic antibody production in laboratory animals. Furthermore, the adjuvant effect of $Al(OH)_3$ for the enhancement of IgE antibody response was studied in various mouse strains (Levine and Vaz 1970). Several other adjuvants have also been employed to potentiate the IgE response. These include bacterial lipopolysaccharides (Perini and Mota, 1973; Newburger et al., 1974; Denneman and Michael, 1976), Bacillus subtilis (Malkiel and Hargis, 1971), Silica (Mancino and Bevilacqua, 1978, 1979; Mancino and Ovary, 1980), bacterial cell wall peptidoglycan and synthetic N-acetylmuramyldipeptide (Ohkuni et al., (1977). However, alum and BP vaccine are still considered the adjuvants of choice. These two adjuvants not only potentiate IgE production but also have enhancing effects on IgG antibody production. Conversely CFA was found ineffective in eliciting IgE responses despite its strong adjuvant activity for IgG antibody response (Mota, 1964; Clausen, 1969; Tada, 1975; Katz, 1978b). On the contrary immunization of mice (Tung et al., 1978) and rats (Tada et

al., 1972) with antigen in CFA resulted in the generation of IgE-selective antigen-nonspecific suppressor T cells. Itaya et al., (1980) showed the suppression of both IgE and IgG immunoglobulin isotypes in animals previously injected with CFA alone, upon subsequent immunization with hapten-carrier conjugate in alum. However, recently IgE production has been reported in a few inbred strains of mice following immunization with soluble antigens in association with CFA (Kudo et al., 1982; Hirano and Ovary, 1983).

2.2 Genetic Control of the IgE Antibody Response:

During the studies done by Revoltella and Ovary (1969); Levine and Vaz, (1970) and Vaz et al., (1971), on the immune responsiveness of various strains of mice, it was revealed that the IgE antibody responses are under genetic control, linked to the H-2 major histocompatibility complex (MHC). Subsequently Levine and Vaz (1972) reported that at least in their experimental system genetic control had only little correlation with the H-2 complex or with the immunoglobulin allotypes. In man Marsh (1976) showed that the extent of the reaginic response appeared more correlated with the total concentration of IgE in the serum than to the control linked to the HLA system. Differences in mounting an IgE response between rats of inbred strains have also been noted by many investigators (Murphey et al., 1976; Pauwels et al., 1979; Yoo and Kuo, 1980).

It was, therefore, suggested that IgE antibody formation is under the genetic control but involves many separate genes and that not all belong to the MHC (Bazin et al., 1981; Bazin and Pauwels, 1982).

2.3 Cellular Interactions in the IgE Production:

It has been well-established that the IgE antibody response is strictly thymus-dependent. Michael and Bernstein (1973) showed that the IgE response cannot be induced in nude mice. The studies with neonatally thymectomized mice further proved the thymus dependency of the IgE formation (Nomoto et al., 1978). Moreover, T-independent antigens DNP-salmonella (Shinohara and Tada, 1974) and DNP-Ficoll (Watanabe et al., 1977; Santomauro and Ovary, 1980) were found incapable of mounting an IgE response in mice. On the other hand a substantial increase in IgE bearing cells in the spleens and lymph nodes of neonatally thymectomized rats was noted following infection by the nematode Nippostrongylus brasiliensis, suggesting that T cells are not required for the generation of IgE B cells (Urban et al., 1977). It was clearly demonstrated that although hapten-specific B cells could be primed with T-dependent or T-independent antigens in athymic (nu/nu) mice, the participation of T helper cells was essential for the development of IgE B-memory cells and for their differentiation into IgE forming plasma cells (Okudaira and Ishizaka, 1975; Urban et al., 1977; Okudaira et al., 1980).

Using the hapten-carrier conjugates as an immunogen the actual mechanism of cellular cooperation for the induction of immune response was studied in laboratory animals. It has been known for many years that the secondary response to a hapten requires the hapten to be presented on the same carrier molecule which was used for the priming. This is known as "carrier effect" (Ovary and Benacerraf, 1963; Katz et al., 1970; Mitchison, 1971; Rajewsky,

1971). This carrier effect was also established for the IgE responses (Tada and Okumura, 1971b; Kishimoto and Ishizaka, 1973; Okudaira and Ishizaka, 1974; Kojima and ovary, 1975).

Collaboration between carrier-primed T helper cells and hapten-primed IgE B-memory cells was demonstrated both by adoptive transfer experiments (Hamaoka et al, 1973, 1974) and in in vitro cultures (Kishimoto and Ishizaka, 1973; Kimoto et al., 1977). DNP-Ascaris (DNP-ASC) primed rabbit mesenteric lymph node (MLN) cells, restimulated in vitro with the same hapten-carrier conjugate, were able to synthesize both anti-DNP IgE and IgG antibodies. However, when DNP-ragweed antigen (DNP-Rag) was used to restimulate MLN cells, no antibody production occured. This study was further extended and it was found that if a supplemental injection of alum-absorbed ragweed antigen was given to DNP-ASC-primed rabbits one week prior to the cell culture, both DNP-ASC and DNP-Rag stimulated IgE in vitro. In contrast when a supplemental injection of ragweed antigen was given in CFA instead of alum no IgE response occured, but IgG antibodies were produced. It was, therefore, suggested that T helper cells for IgE were distinct from the T helper cells for IgG production (Kishimoto and Ishizaka, 1973).

In an in vivo system in mice Hamaoka et al., (1973) did a similar experiment. Hapten-primed B and carrier-primed T cells were co-transferred into x-irradiated recipients and challenged with an appropriate hapten-carrier conjugate. The recipient showed enhanced secondary IgE and IgG responses. When animals were carrier primed in association with CFA, however, no suppression of IgE was observed. In their system, T cells primed with antigen either in CFA or in alum,

collaborated well with hapten-primed B cells to mount IgE and IgG responses. In subsequent studies (Okudaira and Ishizaka, 1973; Newburger et al., 1974; Ishizaka and Adachi, 1976) it was shown that adjuvant was not required for the priming of T helper cells for IgE and IgG antibody responses.

Based on these findings Katz et al., (1974) postulated the presence of distinct sensitivities of IgE and IgG B cells to the regulatory influences of the same T cell populations rather than the existence of different T cells for different Ig classes as suggested earlier by Kishimoto and Ishizaka (1973). Furthermore, cell-free supernatants collected in vitro from the cell primed in vivo with antigen either in alum or in CFA showed the presence of IgG-enhancing factor in both supernatants, while IgE potentiating factor was detected only in alum supernatant (Kishimoto and Ishizaka, 1975).

Kimoto et al., (1977) established an in vitro culture system for mouse spleen cells and confirmed the previous findings reported by Kishimoto and Ishizaka (1973), regarding distinctive helper activity following priming with antigen in CFA or in alum. Mice were carrier-primed with Ascaris in the presence of alum or CFA, their spleen cells were mixed with the same DNP-OA primed cells. Each of these mixtures were stimulated with either DNP-OA or DNP-ASC in vitro. The results obtained clearly showed that T cells stimulated by antigen administered with CFA had helper activity for IgG antibody response but were deficient in providing help for IgE antibody response.

The conflicting results obtained in different laboratories on this subject were considered probably due to the difference in the experimental systems (in vivo vs in vitro studies), rather than due

to species differences (Kishimoto, 1982). In fact, the existence of separate T suppressor (or helper) cells or factors for IgE and IgG does not necessarily preclude the concept of distinct sensitivities of IgE- and IgG-B cells to the same T cell influences.

2.4 Regulation of the IgE Antibody Responses:

Studies done in the last 15 years revealed that the IgE antibody response is regulated by antigen-specific and nonspecific mechanisms. Levine and Vaz (1970) showed that some strains of mice produce a considerable amount of IgG antibody without mounting an IgE response to conventional antigens. Studies done in several laboratories showed that sublethal irradiation or treatment with moderate doses of cyclophosphamide prior to immunization enhanced IgE antibody production in mice (Chiorazzi et al., 1976; Kojima and Ovary, 1976; Schwenk et al., 1979). Results suggested that enhancement of the IgE response was due to depeletion of nonspecific suppressor T cells which exclusively regulate IgE antibody response. Furthermore, studies on the effect of x-irradiation on the production of IgE antibody and other Ig classes in mice revealed that IgE, IgG and IgM antibodies are regulated by separate mechanisms (de Macedo and Catty, 1977). Tung et al., (1978) and Katz and Tung (1978) extended these studies and detected a soluble, antigen-nonspecific factor, capable of suppressing IgE responses from the serum of low IgE responder mice. The suppressive activity of the serum was increased following injection of CFA into the corresponding mice. Subsequently they showed that the serum and ascites of CFA-treated mice contained not only the suppressor factor but also a soluble factor capable of

enhancing the IgE response selectively (Katz et al., 1979). Furthermore, they showed that the suppressor factor could be obtained from both high and low responder strains, and exerted a selective effect on the IgE response across MHC barriers (Katz et al., 1980).

Parasitic infection and elevation of serum reaginic levels in both man and animals is well documented (Cocca, 1931; Johansson et al., 1960; Sadun and Gore, 1970; Jarret, 1974). In rats and mice the most commonly used parasite to potentiate IgE levels are Nippostrongylus brasiliensis (Nb) (Orr and Blair, 1969; Kojima and Ovary, 1975). Trichinella spiralis (Mota et al., 1969; Rivera-Ortiz and Nussenzweig, 1976) and Ascaris suum (Mitchell, 1976). Strejan and Marsh (1971) and Strejan et al., (1973) have shown that Ascaris suum antigens are very potent immunogens for the stimulation of IgE response in comparison to several other soluble protein antigens.

In a series of experiments Ishizaka et al., showed the effect of nonspecific stimulation on IgE production. In an in vitro culture system mesenteric lymph node (MLN) cells of DNP-OA-primed rats cultured with the homologous antigen in the presence of T cells from Nb infected animals showed an enhanced IgE-selective response of the DNP-OA primed cells without affecting IgG forming cells (Suemura and Ishizaka, 1979). The production of IgE potentiating factor by T cells from Nb infected rats was suspected. Subsequently an IgE potentiating factor was isolated by T cells of Nb infected rats (Suemura et al., 1980; Yodoi et al., 1980) and characterized. It was found that the target of IgE potentiating factor was the IgE bearing cell (Suemura et al., 1980). Yodoi et al., (1980) suggested that IgE-potentiating factor was derived from FC_ER^+ T cells.

The production of IgE binding factors was also reported when rats were injected with either CFA or BP vaccine. The factors produced by the MLN cells (Hirashima et al., 1980) and by the splenic lymphocytes (Hirashima et al., 1981c) of CFA treated rats had suppressive activity for IgE. The same suppressive activity was also found in the serum of CFA-treated rats (Hirashima et al., 1980). Though such serum also contained some IgE potentiating factor the activity of the suppressive factor was much greater than the potentiating factor (Hirashima et al., 1980). On the other hand the IgE binding factor produced by the lymphocytes following the treatment of rats with BP (Hirashima et al., 1981a), Pertussigen or Con A (Iwata et al., 1983) had IgE-potentiating activity. Recently the production of IgE binding factors (containing both the enhancing and suppressive activities) by mouse spleen cells following stimulation with homologous IgE and interferon has also been reported (Uede et al., 1983).

It is well-established that IgE antibodies play a crucial role in the pathogenesis of human allergic diseases. Efforts have been made to control and suppress the undersirable production of IgE antibodies. Most of the work on this aspect was directed towards the induction of tolerance in B or T cell compartments, towards induction of antigen-specific T suppressr cells and the induction of anti-idiotypic antibodies.

Tolerance was induced (with or without the induction of T suppressor cells) in several laboratories by the administration of hapten-conjugated to nonimmunogenic copolymers of D-glutamic acid-D-lysine (dGL) or to polysaccharides (Katz et al., 1973; Watanabe et

al., 1977; Moreno et al., 1978; Mutahasi, 1979) or protein antigen coupled to dGL copolymers or polyethylene glycol (Liu et al., 1979; Lee and Sehon, 1978). The studies on the immunosuppressive effect induced by hapten conjugated nonimmunogenic carrier (isologous gammaglobulins) were initially started in Borel's and Schon's laboratories. These conjugates were injected into various animals and a long lasting immunosuppression was demonstrated (Katz and Borel, 1978; Filion et al., 1980).

Liu et al. (1979) studied the effect of OA and ragweed conjugated-dGL administration of IgE production in mice. They were able to show the suppression of primary and secondary IgE responses in such mice but failed to define the mechanism. Neither the induction of tolerance in B_ϵ cells nor the presence of antigen-specific suppressor cells could be confirmed. While using DNP-dGL as tolerogen Katz et al. (1973) demonstrated the complete suppression of primary and secondary anti-DNP antibody responses following immunization with DNP-OA. Furthermore, treatment of immunized animals with DNP-dGL resulted in abrogation of an ongoing antibody response. The mechanism operated via inactivation of hapten-specific B cells without the involvement of T suppressor cells. Similar results were obtained when mice were treated with DNP-conjugated polyvinyl alcohol (DNP-PVA) except in this case inactivation of B cells was also associated with the appearance of hapten-specific suppressor T cells for IgE and IgG antibody classes (Hubbard et al., 1981).

Another approach to regulate the IgE responses to protein antigens involved the manipulation of T cells, which are strictly required for the induction of IgE responses. Administration of urea-

denatured ragweed antigen E (UD-AgE) in primed and nonprimed mice resulted in IgE antibody suppression, while the same dose of native antigen E failed to suppress the antibody response (Ishizaka et al., 1974). In subsequent studies injection of UD-OA into OA-primed mice not only suppressed the response to the priming antigen but also to subsequent injections of sensitizing antigen DNP-OA. Antigen-specific T suppressor cells generated in UD-OA-treated mice were shown to be responsible for the suppression (Takatsu and Ishizaka, 1975, 1976). Lee and Sehon (1978) converted an immunogen OA to nonimmunogen or tolerogen by treating it with polyethyleneglycol (OA-PEG). Basically, Ishizaka's UD-OA and Sehon's OA-PEG contained very similar immunological properties, with respect to the loss of major antigenic determinants and the ability to induce suppressor T cells.

Modulation of the IgE antibody response to BSA by the fragments of the antigen (peptic fragments) (Muckerheide et al., 1977) was reported in mice. This suppression was attributed to the generation of antigen-specific T suppressor cells. When this peptic fragment of BSA was conjugated to homologous mouse gamma globulins and injected to mice it proved more efficient in suppressing IgE to the intact protein than free fragments. This conjugate affected B lymphocytes without T cell involvement (Muckerheide, 1981).

Schwenk et al., (1978) reported an antigen-specific, IgE-selective suppression in $B_6D_2F_1$ mice which were first treated with DNP-liposome and then immunized with DNP-OA. The results were attributed to the possible interaction of the DNP-specific receptors (on the appropriate lymphocytes) with the nonimmunogenic DNP-liposome conjugates. From our laboratory Colby et al., (1980) showed IgE

suppression following treatment of CBA mice with deaggregated OA. The results demonstrated that the T cell dependent tolerance of the IgE antibody response operated via two distinct mechanism, of which only one was provided by suppressor cell function, the other was through T cell tolerance. In a more recent study Hayglass and Strejan, (1983) have reported that pretreatment of CBA mice with glutaraldehyde-polymerized ovalbumin suppressed the induction of IgE antibody response following immunization with native OA. This suppression was antigen-specific, IgE-selective and mediated by T suppressor cells.

Administration of conjugates of ovalbumin-pullulan (Usui and matuhasi, 1979) and ovalbumin-Ficoll (Carter, 1980) also had suppressive effect on the induction of IgE antibody response in mice to the subsequent immunization with native ovalbumin. This suppression in both cases was antigen-specific and IgE-selective and mediated by antigen-specific T suppressor cells.

An antigen-specific IgE selective suppression was shown in BALB/C mice by pretreating them with fatty acid (palmitic acid) modified ovalbumin (OA-Pal) upon subsequent immunization with unmodified antigen OA (Segawa et al., 1981). Pretreatment suppressed both primary and secondary anti-OA IgE responses. Furthermore, treatment of mice with OA-Pal following priming, abrogated an ongoing IgE response. The mechanism was operating via antigen-specific suppressor T cells, generated following treatment with hydrophobically modified antigen.

Recently Chen and Katz (1983) induced IgE class-specific tolerance in both high and low IgE responder mice by neonatal administration of soluble or cell-bound IgE. Their results showed

that high responder mice developed IgE class-restricted tolerance upon repeated neonatal injections of soluble IgE but not by IgE-conjugated splenocytes, while low IgE responder mice exhibited tolerance by either form of IgE injected. It was suggested that non-antigen-specific, isotype restricted tolerance can be induced in the IgE antibody system.

The presence of IgE isotype-specific regulatory T cells have been suggested by many investigators in several experimental systems (Kishimoto et al., 1976; Itaya and Ovary, 1979; Segawa et al., 1981). However, the conclusive evidence for the existance of such cells was provided by Kishimoto et al., (1976) in DNP-mycobacterium-treated BALB/C mice. The findings that priming of animals with carrier antigen included in CFA induced carrier specific helper T cells for other Ig classes excluding IgE antibodies suggested that CFA immunization may be related to the induction of IgE class specific suppressor T cells (Kishimoto and Ishizaka, 1973; Katz et al., 1974; Kimoto et al., 1977). Moreover, Mota (1964) demonstrated that CFA immunization did not induce the IgE response but rather reduced the ability of the same animal to elicit IgE antibody response to the same antigen.

On the basis of these observations, Kishimoto et al., (1976) decided to use Mycobacterium, which is an integral part of CFA as a carrier molecule for a hapten-carrier conjugate (DNP-Myc). Results obtained by these investigators provided strong evidence for the existence of IgE class-specific regulatory T cells in the spleens of DNP-Myc treated BALB/C mice (Kishimoto et al., 1976). These T suppressor cells were hapten (DNP)-specific. This study was extended

further and IgE-selective suppressive effect was demonstrated in an in vitro culture system. Cell free supernatants obtained from the culture of DNP-Myc primed spleen cells after in vitro restimulation with DNP- on a heterologous carrier-pulsed macrophages or DNP-human serum albumin (DNP-HSA)-coupled sepharose, contained the suppressor activity. The suppressive effect of the supernatant was antigen-nonspecific but restricted for IgE Ig class (Suemura et al., 1977), although DNP-specific restimulation was required for its induction. In subsequent studies these factors (IgE-TSF) were further characterized. It was shown that IgE-TSF contained the H-2 gene products and did not exert their functions across H-2 barriers. Moreover, absorption experiments of IgE-TSF with DNP-OA primed B lympocytes and the adoptive transfer experiments revealed that the target cells for the factors are IgE B cells (Kishimoto et al., 1978). Furthermore, DNP-reactive, IgE-selective suppressor T cells were fused with a T lymphoma cell line and a T cell hybridoma secreting IgE class-specific suppressor factor was obtained (Watanabe et al., 1978).

For the direct comparison of the IgE and IgG antibody responses, an IgE-specific reverse plaque forming cell assay was established (Suemura et al., 1981). The IgE selective suppressive effect of IgE-TSF was confirmed by in vitro culture system. Moreover, by specific absorption technique it was shown that IgE-TSF had binding sites for IgE and H-2 gene products (Suemura et al, 1981).

These results suggested the possibility that administration of mycobacterial cells conjugated with allergenic determinants might induce IgE-selective suppressor T cells. Since N-

acetylmuramyldipeptide (MDP) has been known to be the minimum structure required for adjuvanticity in peptidoglycan of bacterial cell walls (Ellouz et al., 1974; Kotani et al., 1975), the effect of pretreatment of DNP-lysine-conjugated MDP on the generation of anti-DNP IgE response to DNP-OA was studied in BALB/C mice (Kishimoto et al., 1979). By using such conjugate they were able to confirm their previous finding (Kishimoto et al., 1976). Instead of DNP, when mice were pretreated with OA-conjugated MDP the suppression was less effective as compared to DNP-MDP. Their results suggested the possibility of the therapeutic application of allergen-conjugated MDP in human atopic diseases.

Recently Kishimoto et al., (1981) induced phosphorylcholine-specific, T15-idiotype bearing T cells in BALB/C mice by injecting them with PC-conjugated Mycobacterium tuberculosis (PC-Myc) and showed the involvement of these T cells in the regulation of IgE antibody response in an antigen-specific manner. Following treatment with PC-Myc mice failed to mount anti-PC IgE response upon immunization with PC-KLH. Mice pretreated with PC-Myc showed suppressed anti-DNP IgE responses only when challenged with PC-KLH-DNP conjugate but not when challenged with DNP-KLH (Kishimoto et al., 1981). In a more recent report (Sugimura et al., 1982), PC-Myc primed splenic T cells were hybridized with T lymphoma cells. Two distinct types of hybridomas were obtained, one was Pc-specific and produced soluble factors with suppressive activity on anti PC IgE and IgG responses while the other was antigen-nonspecific and produced

factors with IgE-selective suppressive effects. Their results suggested the involvement of two distinct subsets of T cells for the expression of the IgE suppression.

This study was continued further and the effect of these two factors was shown on IgE secreting B hybridomas in vitro (Suemura et al., 1983). They showed that the proportion of surface IgE positive cells as well as the number of cytoplasmic IgE positive cells was reduced when IgE secreting hybridoma cells were treated with IgE-selective, T-suppressor factors (TSF). On the contrary the treatment with antigen-specific TSF obtained from PC-specific T hybridomas did not show any inhibitory effect. Moreover, it was shown that IgE-TSF had affinity for the IgE present on the surface of B hybridoma, while IgE-TSF failed to show any suppressive effect when the surface IgE was blocked by anti-IgE antibodies.

Kato and Yamamoto (1982), showed that immunization of C3H/He, C57BL/6, BALB/C and DBA/1 mice with large doses of DNP-conjugated bacillus Calmette-Guérin (DNP-BCG) by i.v., i.p., or s.c. route resulted in the production of IgE antibodies while SJL mice failed to mount such a response. The conflicting findings on the role of CFA in IgE production can perhaps be explained on the basis of the genetic background of the experimental animal.

Among various other approaches which have been applied to abolish the induction or to abrogate an ongoing IgE antibody response was the induction of anti-idiotypic antibodies. Idiotypic anti-idiotypic interactions have been shown to contribute in the regulation of the immune responses in experimental animals (Eichmann and Rajewsky, 1974; Kohler, 1975; Sy et al., 1979). However, the

investigations to regulate the IgE antibody response by anti-idiotypic antibodies were initiated in de Weck's laboratory a few years ago. Studies done with phosphorylcholine (PC), benzylpenicillolyl (BPO) (Imanishi et al., 1975; Blaser et al., 1979, 1980) and with a synthetic antigen L-glutamic acid-L-lysine-L-tyrosine (GAT) system (Dessein et al., 1980) have revealed that IgE antibodies share similar idiotypes with other immunoglobulin (Ig) isotypes. Thus far, experiments done in de Weck's laboratory, both by actively produced or passively administered isologous anti-idiotypic antibodies (against the idiotypes present on BPO and PC binding antibodies), have revealed that the regulation of antibody responses by anti-idiotypic antibodies is a potential way to manipulate the immune response in an antigen-specific manner (Blaser and de Weck, 1982). This could be easier in situations where the response to an antigen is restricted to a certain idiotype. Since IgE antibody is exquisitely sensitive to the positive and negative effects of regulatory mechanisms, its production can be modulated by the procedures which have very little or no effect on other Ig classes. Indeed Blaser et al., showed an intensed suppressive effect on IgE antibody responses (both primary and an ongoing) as compared to other Ig isotypes by anti-idiotypic antibodies (Blaser et al., 1981a; Blaser and de Weck, 1982). In atopic individuals such specific suppression of IgE response is highly desirable. It is hoped that the goal to cure and protect man from allergic diseases will soon be achieved.

3. MATERIALS AND METHODS

3.1 Animals:

Inbred CBA/J ($H\text{-}2^k$) male mice, 8-12 weeks old, were used throughout these studies, except in a few experiments where other strains were compared. These were 8-12 weeks old A/J ($H\text{-}2^a$), B10/SgSn ($H\text{-}2^a$), C57BL/6J ($H\text{-}2^b$), BALB/CJ ($H\text{-}2^d$), DBA/2J ($H\text{-}2^d$), AKR/J ($H\text{-}2^k$) and C3H/HeJ ($H\text{-}2^K$) of both sexes. All mice were purchased from Jackson Laboratories, Bar Harbor, Maine. White New Zealand rabbits weighing 2.5-3 Kg were obtained from M and P Commercial Rabbitry, Ayr, Ontario. Sprague-Dawley female rats (retired breeders) were obtained from Charles-River, Canada, Ottawa, Ontario.

3.2 Antigens:

Crystalline bovine serum albumin (BSA) and twice recrystallized ovalbumin (OA) were purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Keyhole limpet hemocyanin (KLH) was obtained from Biomarine Pacific Supply Co., Venice, California. Bordetella pertussis (BP) vaccine containing 3×10^{10} killed organisms/ml suspension was purchased from the Armand Frappier Institute, Laval, Quebec. Ascaris crude extract (CE) and purified Ascaris allergen, ASC-1 were prepared as described by Hussain et al., (1973).

3.3 Preparation of 2,4-dinitrophenyl Coupled Bordetella pertussis Organisms (DNP-BP):

Killed BP organisms were dinitrophenylated according to Kishimoto et al., (1976). Briefly, 15 ml BP vaccine containing 4.5 x 10^{11} bacteria were centrifuged at 9,000 rpm for 15 min. in a Sorvall RC-2B refrigerated centrifuge, the pellet was resuspended in 0.15 M NaCl (Saline) and the cells were washed with saline at least 5 times by centrifugation (9,000 rpm for 15 min.). The washed organisms were then resuspended in 0.25 ml of 0.1 M Na_2CO_3 and 2.5 µl of 1 Fluoro-2,4 dinitrobenzene (DNFB, Eastman-Kodak Co., Rochester, NY) were added to the cells. The mixture was protected from light and stirred gently at 37°C for one hour. Following dinitrophenylation unbound hapten was removed from the DNP-BP preparation by centrifugation (9,000 rpm for 15 min.) at least 5 times using 0.14 M Na borate boric acid buffer containing 0.15 M NaCl (BBS, pH 8.4). Finally DNP-conjugated BP organisms were suspended in BBS at a concentration of 7.5 x 10^{10} organisms/ml and stored at 4°C. The number of DNP groups/organism was determined by lyzing a small aliquot in boiling 1 N NaOH solution and determining the absorbance at 360nm. Assuming a molar extinction coefficient of 17,500 for DNP various DNP-BP preparations were found to contain 3-15 x 10^6 DNP groups/cell and each ml of suspension contained 3.75 mg dry weight (50 µg/10^9 DNP-BP). Other hapten-BP (DNP-BP) conjugates were prepared essentially by the same method but using different hapten:BP ratios for substitution.

3.4 Preparation of Other Hapten-carrier Conjugates:

Other proteins were substituted according to Eisen et al., (1958) using four times crystallized dinitrobenzene-sulfonic acid -Na salt (DNBS, Eastman-Kodak). The following conjugates were obtained: DNP_2-CE, DNP_3OA, DNP_{16}-BSA, DNP_{29}-BSA, DNP_2-ASC-1 and DNP_{75}-KLH. The subscripts refer to the average number of moles of DNP/mole of carrier assuming a molecular weight of 40,000 for OA, 72,000 for BSA, 800,000 for the dissociated form of KLH and 17,000 for ASC-1. In the case of CE the subscript represents moles of DNP x 10^7/mg carrier.

3.5 Immunization:

Unless otherwise stated, mice were injected intraperitoneally (i.p.) with a total volume of 0.5 ml of antigen either in saline or absorbed on Al $(OH)_3$ gel (alum). Usually 2 mg alum were used for each injection. A booster injection was given by the same route 4 weeks following the first injection. Alum was prepared from AlK $(SO_4)_2$ and NaOH according to Chase (1967). The stock preparation contained 10 mg dry weight/ml. In some cases, prior to immunization the antigen was emulsified in complete Freund's adjuvant (CFA) or in incomplete Freund's adjuvant (IFA, Difco Laboratories, Detroit, MI). Mice were bled by cardiac puncture under ether anesthesia. The blood from all animals in an individual group (usually 4 mice) was pooled and sera were stored at -20°C until needed.

Rabbits were immunized repeatedly with 5 mg of protein antigen emulsified in CFA in a final volume of 1 ml. The first injection was given intradermally (i.d.) at multiple sites in the nuchal area. Subsequently, i.d. and intramuscular (i.m.) injections were given

alternately at 2 weeks intervals for the first 2 months and then once a month. Rabbits were bled through an ear vein and the final bleeding was by cardiac puncture under Na-pentothal anesthesia. The sera were stored at -20°C.

3.6 Preparation of Antisera:

(a) Mouse anti-TNP-KLH serum:

Mice were immunized with 100 µg TNP-KLH emulsified in CFA and were boosted two weeks later with the same amount of antigen in IFA. The third injection was given in saline 2 weeks following the secondary immunization. Mice were bled one week after the third injection. The sera were pooled and the amount of anti-TNP antibodies was determined by the quantitative precipitin assay. It contained 2.54 mg anti-TNP antibodies/ml. The anti-serum was stored in small aliquots at -20°C and served as an anti-DNP IgG standard for radioimmunoassays (RIA).

(b) Rabbit Anti-mouse IgG (RAMG):

Mouse IgG was prepared from pooled normal mouse serum by ammonium sulfate precipitation followed by DEAE cellulose chromatography according to Campbell et al., (1970). The purity of mouse IgG was determined by immunoelectrophoresis against rabbit-anti-mouse serum. Rabbits were immunized with pure mouse IgG and bled as described earlier (section 3.5). The sera were pooled and stored in separate aliquots at -20°C until needed.

(c) Rabbit Anti-mouse Ig (RaMIg):

A crude mouse Ig preparation was obtained from pooled normal mouse serum by ammonium sulfate precipitation, according to Campbell et al., (1970). The rabbits were immunized and bled as described earlier (section 3.5). The sera were pooled and stored at -20°C in separate aliquots.

3.7 Preparation of Affinity Columns:

Mouse IgG, DNP-BSA or OA were coupled separately to cyanogen bromide (CN-Br) activated Sepharose-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the procedures recommended by the manufacturer. Briefly, the freeze-dried material was washed and reswelled in 1 mM HCl, washed with 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl (pH 8.0) and mixed with the protein to be conjugated. Tubes containing the mixture were rotated end-over-end overnight at 4°C. The remaining active groups were blocked by rotating the Sepharose in a tube containing 1 M monoethanolamine in 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl (pH 9.0). The excess blocking agent was then washed away with 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl (pH 8.0). The immunosorbents were then cycled in successive washes of 0.1 M borate buffer containing 0.5 M NaCl (pH 8.0), followed by 0.2 M Na-acetate buffer containing 0.5 M NaCl, (pH 4.0). The cycle of washing with borate and acetate buffers was repeated 5 times, prior to resuspending the immunosorbents in borate buffer (0.1M with 0.5 M NaCl, pH 8.0). Sodium azide was added to a final concentration of 0.04%, the immunosorbents were stored at 4°C, and used within 4 months after conjugation.

3.8 Preparation of Sephadex G-100 Column:

Sephadex G-100 (Pharmacia Fine Chemicals) was swollen in distilled water, at room temperature for a week, with frequent changes of water. Swollen beads were then washed 5-6 times in PBS (0.15 M, pH 7.4) and deaerated. A column of 2.5 x 86 cm size was prepared and equilibrated with PBS prior to use.

3.9 Fractionation of Serum on Sephadex G-100 Column:

Sephadex G-100 column prepared and equilibrated with 0.15 M PBS as mentioned earlier (section 3.8) was calibrated with 10 mg of each, BSA, OA, and cytochrome C. A sample of serum obtained from DNP-BP treated, DNP-OA injected, IgE suppressed mice, after removing anti-DNP and anti-OA antibodies (as confirmed by RIA) was applied to the column and eluted with 0.15 M PBS. Fractions approximating the molecular weight of the markers and void volume were collected and concentrated to the original volume by ultrafiltration using YM2 and YM10 membrane (Amicon Corp., Lexington, Mass.) depending on the molecular size of the fraction to be concentrated. All fractions were dialyzed against PBS (0.15 M, pH 7.4), sterilized by filtration (Millipore 0.45 µm filter) and stored at -20°C until used.

3.10 Preparation of Affinity Purified Antibodies:

Rabbit anti-mouse IgG (RAMG) was affinity purified by passage through mouse IgG-sepharose 4B and mouse anti-DNP antibodies were purified by DNP-BSA sepharose 4B immunosorbents. The relevant serum was mixed with the antigen-coupled sepharose beads in a 15 ml centrifuge tube, and tube was rotated end over end for 2 hrs. at room

temperature. The effluent was collected by filtration. The immunosorbent was washed thoroughly with 0.1 M Borate buffer containing 0.5 M NaCl (pH 8.0) until no protein was detected in the effluent (OD 0.00 at 280 nm), and then bound antibodies were eluted with 0.2 M glycine-HCl (pH 2.3). The eluate was immediately neutralized (pH 7.0) by the addition of 1 N NaOH and dialyzed against PBS (0.15 M, pH 7.4). The protein in the eluate was then concentrated by ultrafiltration (Amicon, YM10 membrane) whenever required.

3.11 X-irradiation:

Depending on the protocol required, mice were exposed to 250 R, 660 R or 960 R from a Gammacell-20 irradiator (Atomic Energy of Canada, Ltd., Ottawa).

3.12 Cyclophosphamide Treatment:

Three days prior to the immunization mice were injected i.p. with either 60 or 150 mg/Kg body weight of cyclophosphamide base (Procytox, Horner, Montreal) freshly dissolved in distilled water.

3.13 Preparation of Single Cell Suspension:

Spleens from donor mice were collected aseptically in Hanks balance salt solution (BSS), minced and ground gently with a teflon pestle in 17 x 100 mm sterile tube (Falcon Plastics, Oxnard, CA). The large debris were allowed to settle and the supernatant was transferred to a new tube and was centrifuged at 1000 rpm for 5

minutes at 10°C. The cells were then washed twice in BSS, resuspended in an appropriate medium and the viability was determined by trypan blue exclusion test (TBET).

3.14 Purification of T Cells by Ig-coated Dishes:

T cells from the spleens of primed or nonprimed donors were isolated by the technique described by Mage et al., (1977) and modified by Wysocki and Sato (1978). Briefly, sterile 15 x 100 mm polystyrene bacteriological Petri-dishes (Fisher Scientific Co., Don Mills, Ont., Cat. no. 8-757-12) were coated with 350 µg affinity purified RAMG in 10 ml of 0.05 M Tris (pH 9.5) by incubation at room temperature for 40 minutes. The buffer was decanted and the dishes were washed 4 times with 0.01 M PBS (pH 7.4) and finally with 20 ml of PBS containing 1% fetal calf serum (FCS, Microbiological Associates, Bethesda, MD). The RBCs from the spleen cell suspension were removed by 0.85% ammonium chloride and 3 x 10^7 nucleated cells were suspended in 3 ml PBS containing 5% FCS and poured onto the antibody coated dishes. The plates were incubated at 4°C, on a level surface for 70 minutes. Following 40 min of incubation, unattached cells were redistributed by tilting and swirling the plate. After 30 more min the nonadherent cells were collected by decanting the supernatant. Plates were washed twice with PBS containing 1% FCS, the supernatant and washings were pooled and centrifuged at 1000 rpm for 5 min. The cells in the pellet were washed twice with BSS. The viability of the recovered cells was determined by trypan blue

exclusion. More than 95% of the cells were viable as determined by TBET. The cells were stimulated with mitogens to determine their purity, and 95% pure T cells were isolated by this method.

3.15 Depletion of T Cells:

Spleen cell suspensions were depleted of T cells by treatment with monoclonal anti-Thy 1.2 (Becton-Dickinson Co., Mountainview, CA) and complement. Complement (guinea pig serum) was detoxified prior to use by absorbing each ml with 10 mg agarose and 0.1 ml packed mouse RBC for 60 min. at 4°C. Spleen cells were suspended in BSS at a concentration of 2×10^7 cells/ml and 7 µg of anti-Thy 1.2 antibodies were added to each ml of the cell suspension. Cells were kept on ice for 45 min and then centrifuged at 1000 rpm for 5 min. Pelleted cells were washed once in BSS by centrifugation (1000 rpm for 5 min.) and complement was added to the cell suspension at a final dilution of 1:10 in BSS. Cells were incubated at 37°C in a water bath for 30 min to allow (complment mediated) lysis of T cells to occur. The cell suspension was then centrifuged at 1000 rpm for 5 min, the cells in pellet were washed twice and suspended in an appropriate medium. This treatment killed 35-40% of the spleen cells.

3.16 Stimulation of Spleen Cells by Mitogens:

Enriched T and B cell populations were stimulated with mitogen to determine their purity (Wysocki and Sato, 1978). Cells were suspended at a concentration of 5×10^6/ml in RPMI 1640 medium (Gibco, Grand Island, NY), containing 10% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml fungizone

and were grown at a concentration of 5×10^5 cells/well in the presence of either 2.5 µg *E. coli* 0128:B12 lipopolysaccharide (LPS, Difco) or 0.25 µg concanavalin A (ConA, Sigma Chemical Co., St. Louis, MO), in a total volume of 0.2 ml/well, in a multiwell U bottom microtiter tissue culture plate (Linbro Scientific, Inc., Hamden, CT) for 48 hrs. Following incubation 1 µCi of [^{3}H]-thymidine (New England Nuclear, Boston, MA) was added to each well, and cells were harvested after a total of 72 hrs. using an automatic cell harvestor (Otto Hiller Co., Madison, WI). The radioactivity was counted in a beta scintillation counter, LKB model Wallac 1215 Rackbeta.

3.17 Adoptive Transfer:

Unless otherwise stated, at results, single cell suspensions from primed or normal donors were transferred into normal or x-irradiated syngeneic recipients i.v. In co-adoptive transfers, recipient mice received mixture of cells from more than one type of donors. The mice were challenged with an appropriate antigen in alum, within 2-3 hrs. following cell transfer.

3.18 Passive Transfer of Serum:

Mice were injected i.v. with various volumes of serum or serum fractions 12 hrs. before and 12 hrs. after immunization with 2 µg DNP-OA in alum.

Immunized mice received 0.5 ml serum or serum fractions in a single dose, 10 days after antigen priming.

3.19 In Vitro Treatment of Spleen Cells with Serum or Serum Fractions:

Spleen cells obtained from normal donors were suspended at a concentration of 10^7/ml in RPMI 1640 medium (Gibco) containing 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml fungizone. The medium was supplemented with 6% mouse serum to be tested. Two ml of the cell suspension were cultured in each well of a 6 well tissue culture plate (Costar Division, Cambridge, MA). The plates were incubated at 37°C in a humidified atmosphere for 18 hrs. Cells were harvested, washed twice in RPMI 1640 medium without serum by centrifugation at 1000 rpm for 5 min. and resuspended in RPMI 1640 medium (without serum). The cell viability was determined by TBET (45-50% cells were found alive). The (in vitro treated) cells were adoptively transferred into x-irradiated (660R) syngeneic recipients.

3.20 Iodination of Protein:

DNP_{16}-BSA was labelled with carrier-free Na ^{125}I (Amersham/Searle, Arlington Heights, IL) by the chloramine T method of Yagi et al., (1963). Thus 1 mg of DNP_{16}-BSA was labelled with 0.5 mCi ^{125}I. Unbound iodine was removed by Sephadex G-25 gel filtration followed by 24 hr. dialysis against BBS. This iodinated antigen was used in the determination of anti-hapten (anti-DNP) IgG antibodies by radioimmunoassay (RIA).

3.21 Protein Determination:

Whenever required the amount of protein in solution was determined by the method described by Lowry et al., (1951).

3.22 Determination of Antibodies:

(a) IgE:

Both anti-hapten and anti-carrier mouse IgE antibodies in pools of sera from 4 mice were measured by passive cutaneous anaphylaxis (PCA, Ovary et al., 1975) reaction. The test was performed on female Sprague-Dawley rats. Sera were serially diluted in physiological saline and 0.1 ml volumes were injected i.d. into a shaved area of the back. Fourty-eight hrs. after sensitization, antigen-specific PCA reactions were elicited by i.v. injection of 2 mg of antigen in 1 ml of 1% Evan's blue dye in saline. The reaction was read 20-30 minutes following challenge and results were reported as reciprocal of the highest serum dilution giving a blue area of 5 mm diameter or more. The titers were finalyzed after repeating the PCA on a second rat.

(b) IgG:

The amount of anti-DNP IgG was determined by fluid phase double antibody radioimmunoassay (RIA, Strejan and Surlan, 1977). An anti-TNP-KLH serum containing 2.54 mg anti-TNP antibody/ml was used as standard.

(c) Anti-idiotypic Antibodies (aId):

All assays were carried out in 96 well polyvinyl coated microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA), as described by Geha (1982) with slight modifications. Preliminary titrations were carried out with the aim of establishing the amount

of idiotype (Id) required to bind approximately 75% of 20 ng ^{125}I-DNP-BSA. The source of Id was affinity purified anti-DNP antibody isolated from sera of mice immunized with both DNP-BP and DNP-OA as explained in "Results". Briefly 100 µl volumes of serial dilutions of Id were made in 0.01 M PBS pH 7.4 and were placed in the wells of microtiter plate. The plate was allowed to stand at room temperature for 3 hrs., the wells were washed 3 times with 0.01 M PBS containing 1% BSA (RIA buffer). To each well were then added 20 ng ^{125}I-DNP-BSA in 100 µl volumes and the plate was again left at room temperature for additional 3 hrs. Finally the contents of the wells were discarded, the wells were washed 3 times with RIA buffer, were dried, cut out and counted in a gamma scintillation counter. The wells coated with 1.15 µg Id bound 80% of the ^{125}I-DNP-BSA added. This amount of Id was used to coat the wells in subsequent determinations.

The putative source of anti-Id antibodies was serially diluted in RIA buffer and 100 µl volumes of each dilution were added to Id-coated wells. A parallel series of dilutions of normal mouse serum served as the negative control. After a 3 hr. incubation the wells were washed 3 times with RIA buffer and 20 ng ^{125}I-NDP-BSA were added to all wells. The plate was incubated, wells were washed, dried and counted as described earlier. The results were expressed as % inhibition of ^{125}I-DNP-BSA binding to Id following reaction with aId.

3.23 Statistical Analysis:

PCA titers and IgG antibody levels were logarithmically transformed and analyzed by a student's t test for differences between 2 means or by multi-factor analysis of variance without replication (Zar, 1974). Differences between groups giving "p" values smaller than 0.05 are statistically significant. Otherwise, 4-fold differences or greater in PCA titers were considered statistically significant (Newburger et al., 1974).

4. RESULTS

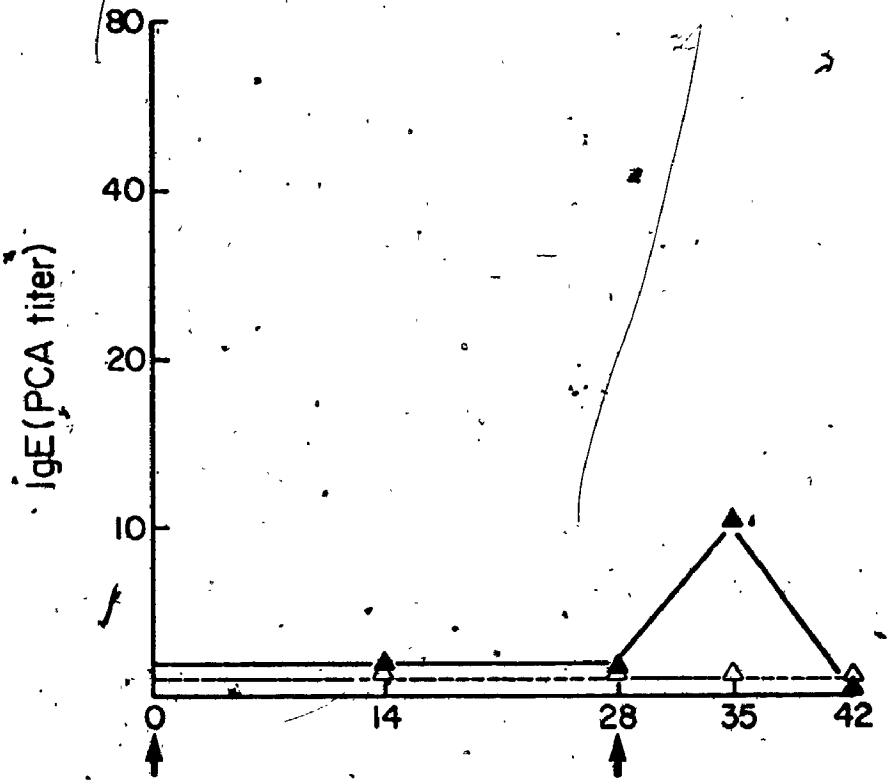
4.1 Immunogenicity of DNP-BP Conjugate:

To evaluate the ability of DNP-BP conjugates to stimulate anti-hapten IgE and IgG antibody responses, groups of CBA mice were immunized with 10^9 DNP-BP organisms (equivalent to 50 µg dry weight), either in saline or mixed with alum. The animals were boosted 28 days later under identical conditions of antigen, dosage, route and composition, as defined for primary injection, and were bled 14 and 28 days after priming and 7 days after booster (day 35). The results (Fig. 1) indicate that immunization with DNP-BP either in saline or mixed with alum failed to stimulate detectable serum levels of anti-DNP IgE antibodies after primary stimulation. Occasionally low PCA titers were recorded in sera obtained 7 days after secondary immunization, which never exceeded 1:80. In contrast, anti-DNP IgG levels were elevated, especially in the group that received DNP-BP in alum. PCA reactions for the detection of anti-BP IgE antibodies were negative regardless of whether the BP preparations used for challenge were intact or were ultrasonically disintegrated to permit better access of the antigen to the mast cell (not shown).

4.2 Influence of Dose and Hapten/Carrier Ratio on IgE Responses:

Results obtained in the previous experiment (Fig. 1) demonstrated the inability of 10^9 DNP-BP conjugates with a hapten/carrier ratio of 3×10^6 DNP groups/BP organism, to mount significant primary and secondary anti-DNP IgE responses in CBA mice.

Figure 1. Antibody production to DNP-BP in CBA/J mice. Mice were immunized with 10^9 DNP-BP on day 0 and 28 (arrows). IgE anti-DNP antibodies are expressed as reciprocal of PCA titer following challenge with 2 mg DNP-BSA in 1 ml 1% Evan's blue. IgG anti-DNP antibodies are expressed in µg/ml serum. △, mice immunized with DNP-BP alone, ▲, mice immunized with DNP-BP and 2mg alum.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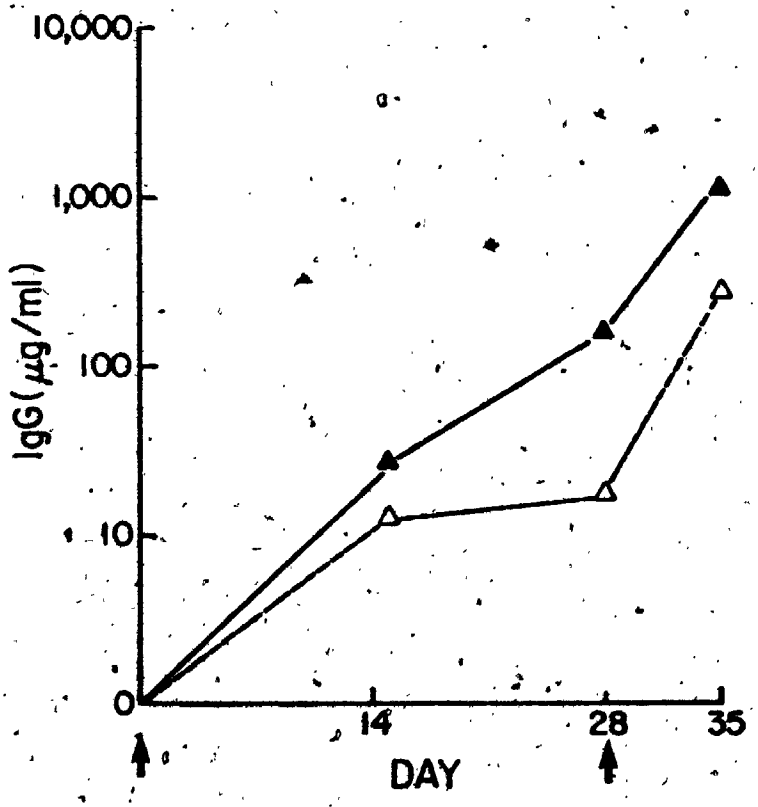
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14
28
35
DAY

In the following experiments, groups of mice were immunized with varying amounts of DNP-BP conjugates (10^8, 10^9 and 10^{10}) in alum (Table I) or with 10^9 DNP-BP at increasing hapten/carrier ratio (3.67 x 10^5, 3.3 x 10^6, 2.9 x 10^7 and 6.9 x 10^7 DNP groups/BP organism) in alum (Fig. 2). The animals were boosted 28 days following primary immunization under the same conditions (dosage, route and composition) as those used for the primary immunization, and were bled on days 14 and 35. A weak anti-DNP IgE secondary response was detected in the group immunized with 10^{10} DNP-BP conjugates (PCA titer 1:80, Table I), while the group primed and boosted with 10^9 DNP-BP showed a PCA titer of 1:10 on day 35 (7 days after booster). The group immunized with 10^8 DNP-BP was unable to mount any detectable anti-DNP IgE responses. None of the groups produced anti-DNP IgE antibodies after primary immunization. On the other hand, anti-DNP IgG responses were obtained in all groups and were directly proportional to the dose of antigen injected. Immunization with 10^9 DNP-BP at various hapten/carrier ratios again demonstrated the failure of this antigen to stimulate high and sustained anti-DNP IgE antibody levels, regardless of the DNP/BP ratio (Fig. 2). Again, anti-DNP IgG primary and secondary responses were obtained in all groups.

4.3 Antibody Responses of Various Inbred Strains of Mice to Immunization with DNP-BP:

The failure of DNP-BP to stimulate IgE antibody production could be explained by an intrinsic defect of this immunogen to trigger IgE B cells; otherwise a clear pattern of high and low responsiveness

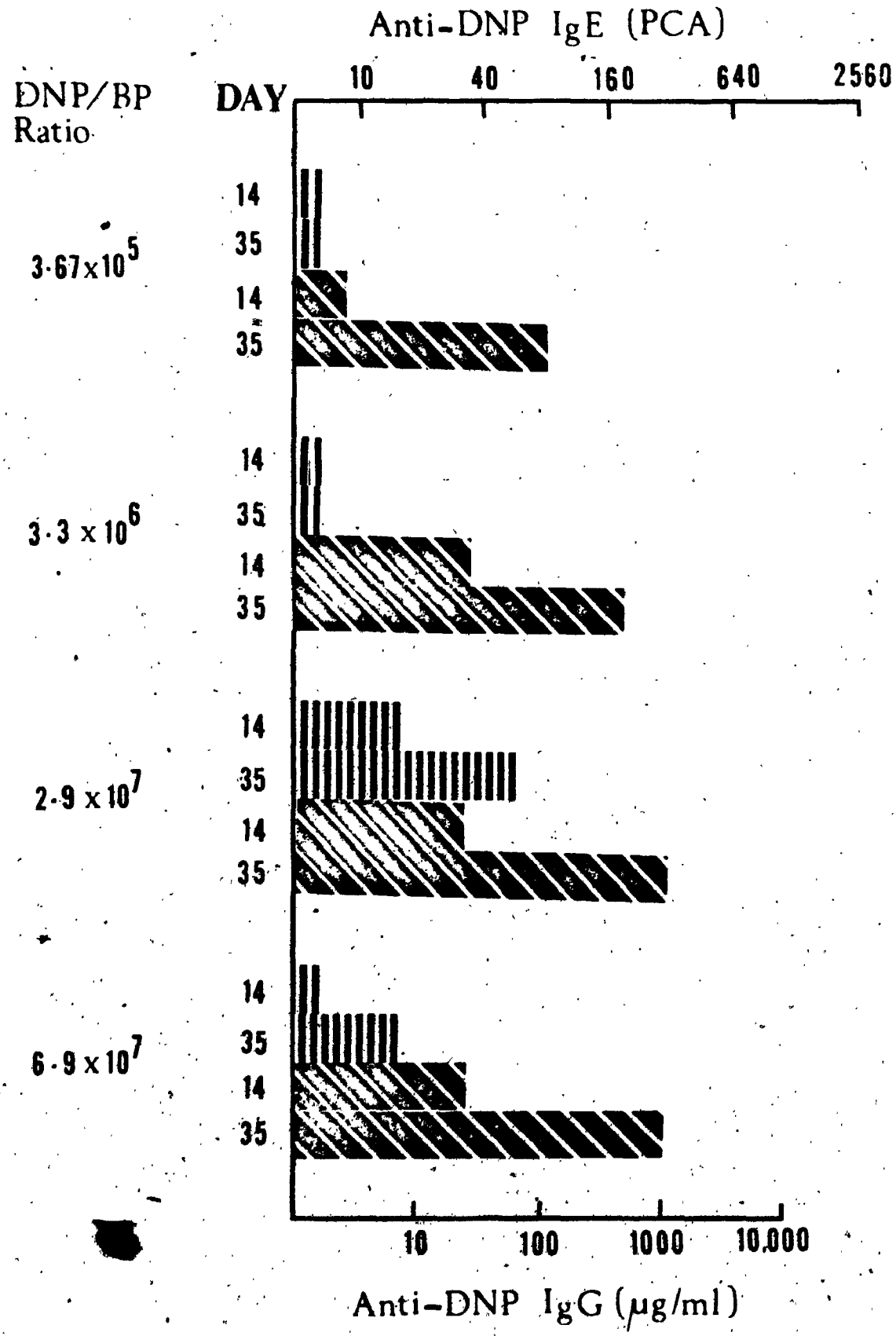
TABLE I

Primary and secondary IgE and IgG anti-DNP responses to various amounts of DNP-BP conjugates.

Group	Immunization[a]	Anti-DNP IgE (PCA titer) on day		Anti-DNP IgG (μg/ml) on day	
		14	35	14	35
1	10^8 DNP-BP (A1)	<10	<10	<1	6
2	10^9 DNP-BP (A1)	<10	10	43	960
3	10^{10} DNP-BP (A1)	<10	80	119	3150

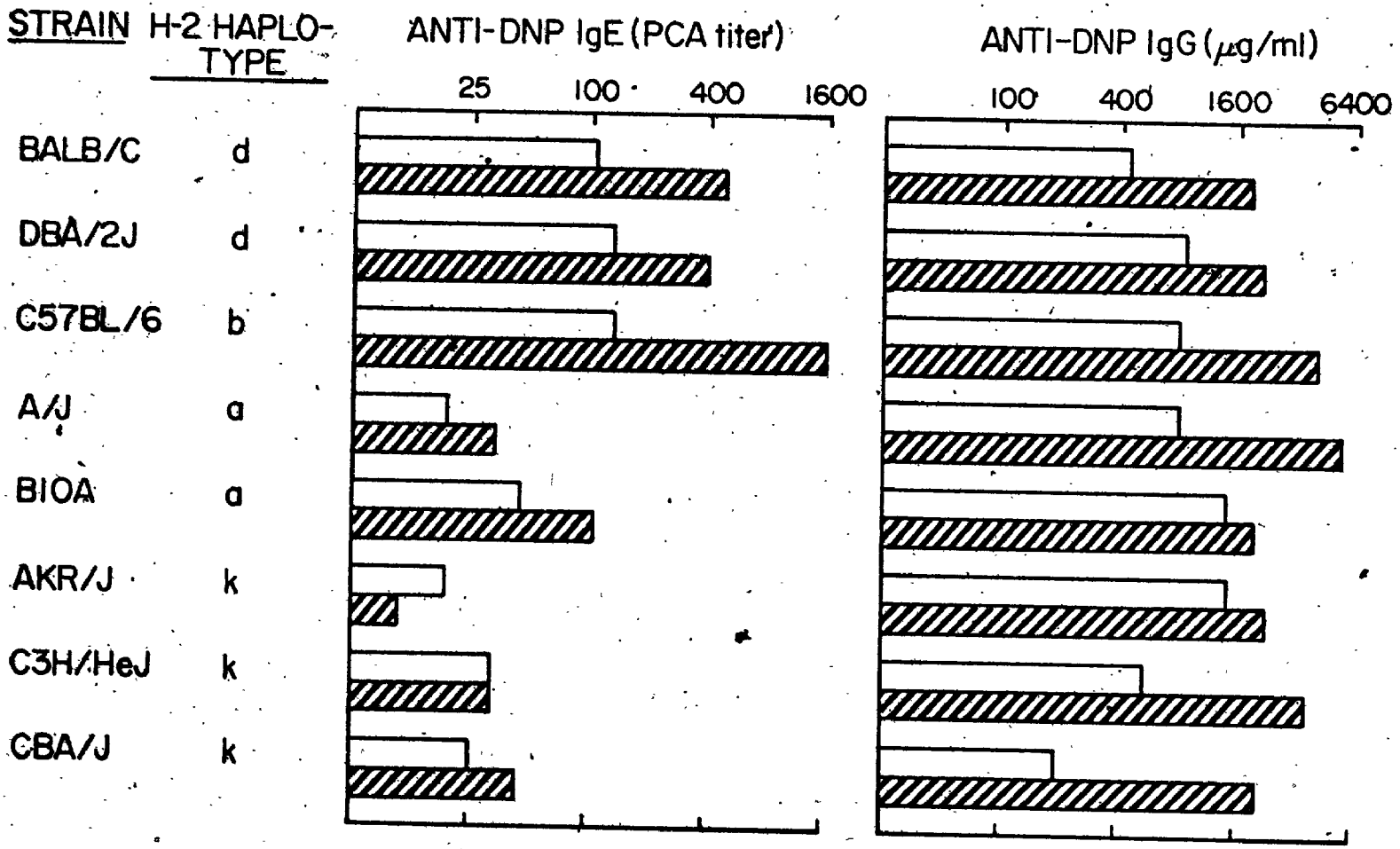
[a] Groups of mice were immunized i.p. with varying numbers of DNP-BP in alum on days 0 and 28.

Figure 2. Primary and secondary anti-DNP responses to DNP-BP at various hapten/carrier ratios. Mice were immunized with 10^9 DNP-BP alum on days 0 and 28 and were bled on days 14 and 35 after primary immunization. The numbers represent groups of DNP/BP organism. ▥, PCA titer, ▨ IgG µg/ml.


Anti-DNP IgE (PCA)
10
40
160
640
2560
DNP/BP Ratio
DAY
14
35
3·67x10^5
14
35
14
35
3·3 x 10^6
14
35
14
35
2·9 x 10^7
14
35
14
35
6·9 x 10^7
10
100
1000
10,000
Anti-DNP IgG (μg/ml)

7

Figure 3. Antibody response of various inbred mouse strains following immunization with DNP-BP in alum. Mice from various strains were immunized with 10^9 DNP-BP in alum on days 0 and 28. Anti DNP-IgE and IgG levels were detected on day 14 (☐) and 35 (▨).


STRAIN
H-2 HAPLO-TYPE
ANTI-DNP IgE (PCA titer)
25
100
400
1600
ANTI-DNP IgG (μg/ml)
100
400
1600
6400
BALB/C d
DBA/2J d
C57BL/6 b
A/J a
BIOA a
AKR/J k
C3H/HeJ k
CBA/J k

among inbred mouse strains carrying different H-2 haplotypes would indicate that DNP-BP is not deficient in stimulating IgE antibody responses. The results presented in Fig. 3 show that mice from all strains studied responded with excellent anti-DNP IgG levels, whereas the IgE antibody response showed a clear strain-dependent distribution. Mice of the $H\text{-}2^a$ and $H\text{-}2^k$ haplotypes responded poorly, with a PCA titer of 1:80 or lower and with secondary responses never exceeding significantly those obtained after primary immunization. On the other hand mice of the $H\text{-}2^b$ and $H\text{-}2^d$ haplotypes were good responders.

These results indicate that the inability of CBA mice to respond to DNP-BP with sustained and boosterable IgE antibody production can not be attributed to an intrinsic lack of immunogenicity of DNP-BP with respect to IgE antibody production.

4.4 Effect of DNP-BP Pretreatment on Primary and Secondary IgE Responses to DNP-OA:

The ability of several mouse strains to produce high levels of anti-DNP IgE antibodies following immunization with DNP-BP (Fig. 3), suggested that the failure of CBA/J strain to respond to similar immunization protocols was due to an IgE-selective suppression. In order to test this assumption, CBA/J mice were pretreated with 10^9 DNP-BP organisms once, (day -14), or 3 times (days -16, -14, -12), and were then immunized with 2 µg DNP-OA in alum on days 0 and 28. Control mice received no pretreatment, 3 injections of 50 µg DNP-ASC-1 or 3 injections of 10^9 unsubstituted BP, followed by the conventional DNP-OA immunization. The results are presented in Fig.

4. It can be seen that all mice pretreated with DNP-BP or DNP-ASC-1 had high anti-DNP serum levels as early as 7 days after immunization with DNP-OA in alum while the non-pretreated group mounted the expected primary IgE responses (PCA titer 1:400) starting on day 14. On the other hand, the anti-DNP IgE levels of the groups pretreated either once or 3 times with DNP-BP were significantly reduced from day 14 onward, when compared to the controls. The IgE anti-DNP levels remained significantly reduced even after booster with DNP-OA alum (day 28). Both primary and secondary anti-DNP IgG responses were high in all groups. The IgG responses determined on day 14 were significantly higher in all DNP conjugate treated groups (DNP-BP or DNP-ASC-1) than in untreated and BP treated groups. Following booster with DNP-OA the untreated group showed a sharp increase in anti-DNP IgG levels while the groups treated with DNP-BP or DNP-ASC-1 had anti-DNP IgG levels that were not significantly higher than those found after the primary immunization with DNP-OA. The relatively low anti-DNP IgG levels in the BP-treated group cannot be explained at the present time.

These results suggested that administration of DNP-BP once or 3 times generated an effective suppressive mechanism(s) responsible for the drastic decrease of anti-DNP IgE levels while a similar treatment with DNP-ASC-1 failed to generate such a mechanism.

To demonstrate the antigen specificity of the suppressive mechanism(s) mice were pretreated 3 times with DNP-BP (days -16, -14, -12), and were immunized with 2 µg OA in alum on days 0 and 28. Control mice remained untreated but were primed and boosted with 2 µg OA in alum on days 0 and 28 respectively. The results presented in

Figure 4. Suppression of the IgE antibody response after treatment with DNP-BP. Groups of CBA/J mice treated i.p. as indicated below were immunized with 2 μg DNP-OA alum on days 0 and 28 (arrows). Anti-DNP antibodies were detected 7, 14, 21, 28 and 35 days following primary immunization.

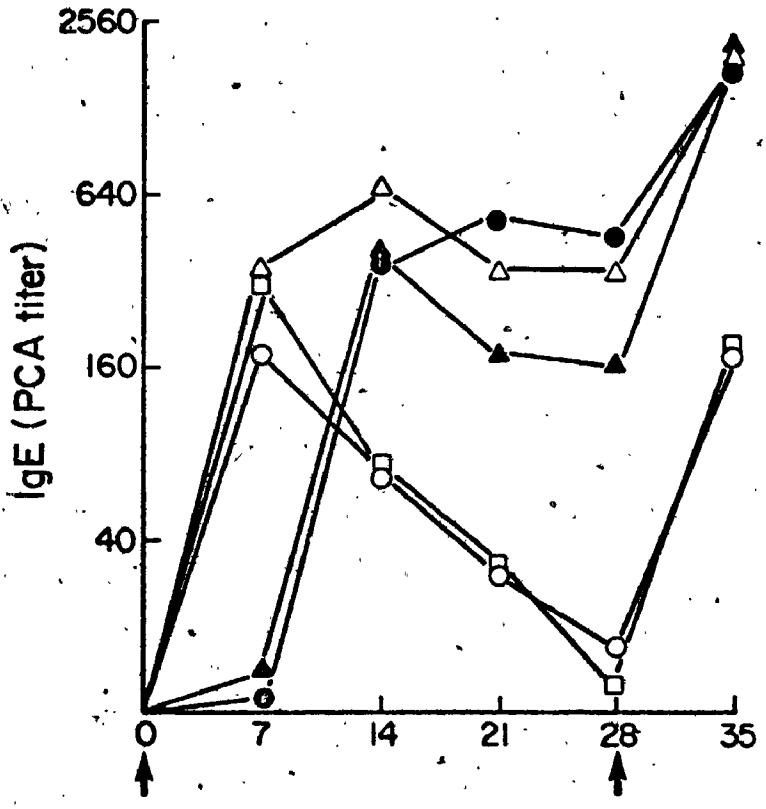
● , no pretreatment

□ , 10^9 DNP-BP on days -16, -14, -12

▲ , 10^9 BP on days -16, -14, -12

△ , 50 μg DNP-ASC-1 on days -16, -14, -12

○ , 10^9 DNP-BP on day -14


2560
640
160
40
IgE (PCA titer)
0
7
14
21
28
35

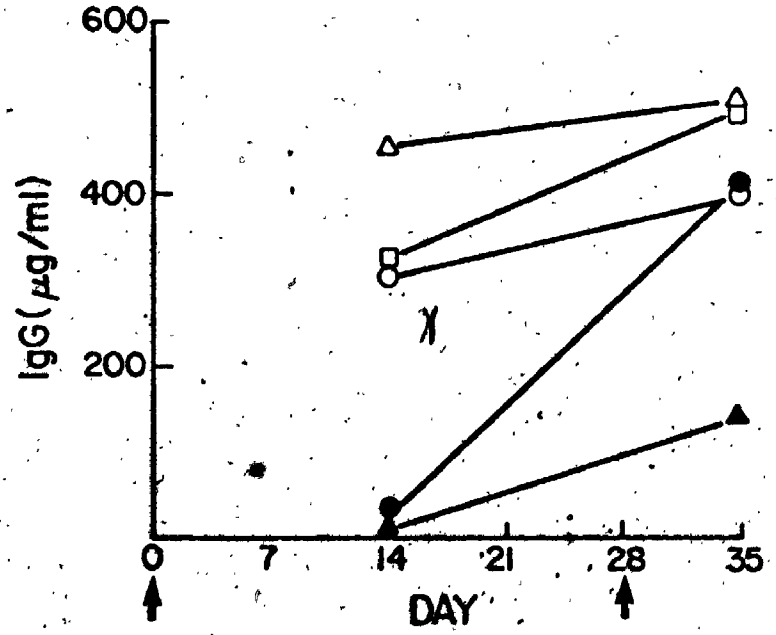

600
400
200
IgG(μg/ml)
0
7
14
21
28
35
DAY

TABLE II

Effect of pretreatment with DNP-BP on the immune response against an irrelevant antigen (OA).

Group	Pretreatment[a]	Anti-OA IgE Antibodies (PCA titer)[b] on day 14	21	28	35
1	10^9 DNP-BP (day -16, -14, -12)	800	400	800	2000
2	None	1000	400	400	4000

[a] Mice were pretreated i.p. with DNP-BP or were left untreated.

[b] On day 0 and 28 all mice were immunized with 2 µg OA in alum and anti-OA IgE antibodies were determined on the days shown.

TABLE III

Immune responsiveness of IgE suppressed mice against an irrelevant antigen (KLH).

Group	Pretreatment	Anti-KLH IgE (PCA titer) on day 35	42	49
1	10^9 DNP-BP (day -16, -14, -12) 2 µg DNP-OA (A1) (day 0)	80	40	80
2	50 µg DNP-CE (day -16, -14, -12) 2 µg DNP-OA (A1) (day 0)	40	40	80
3	2 µg DNP-OA (A1) (day 0)	80	80	40
4	None	80	40	80

Mice were immunized on day 21 with 5 µg KLH in alum. Anti-KLH IgE levels were determined on day 35, 42 and 49 after immunization.

Table II demonstrate that there is no significant difference between the PCA titers of treated and non-treated groups. These results indicate that the suppressive mechanism is antigen (DNP)-specific.

The antigen-specificity of DNP-BP-induced suppression was further demonstrated by immunizing mice with an irrelevent antigen, at the time when the suppression was approaching maximum. Groups of mice were treated with 10^9 DNP-BP on days -16, -14, and -12 and were immunized with 2 µg DNP-OA in alum on day 0. Control groups were treated with DNP-CE and DNP-OA, with DNP-OA alum or were left untreated. On day 21 all mice were immunized with 5 µg KLH in alum.

The results presented in Table III show that all groups had similar anti-KLH PCA titers regardless of whether mice were pre-treated or not. The results clearly show that even under conditions of maximal suppressive activity (day 21) the suppressive mechanism(s) generated by DNP-BP administration remained antigen-specific.

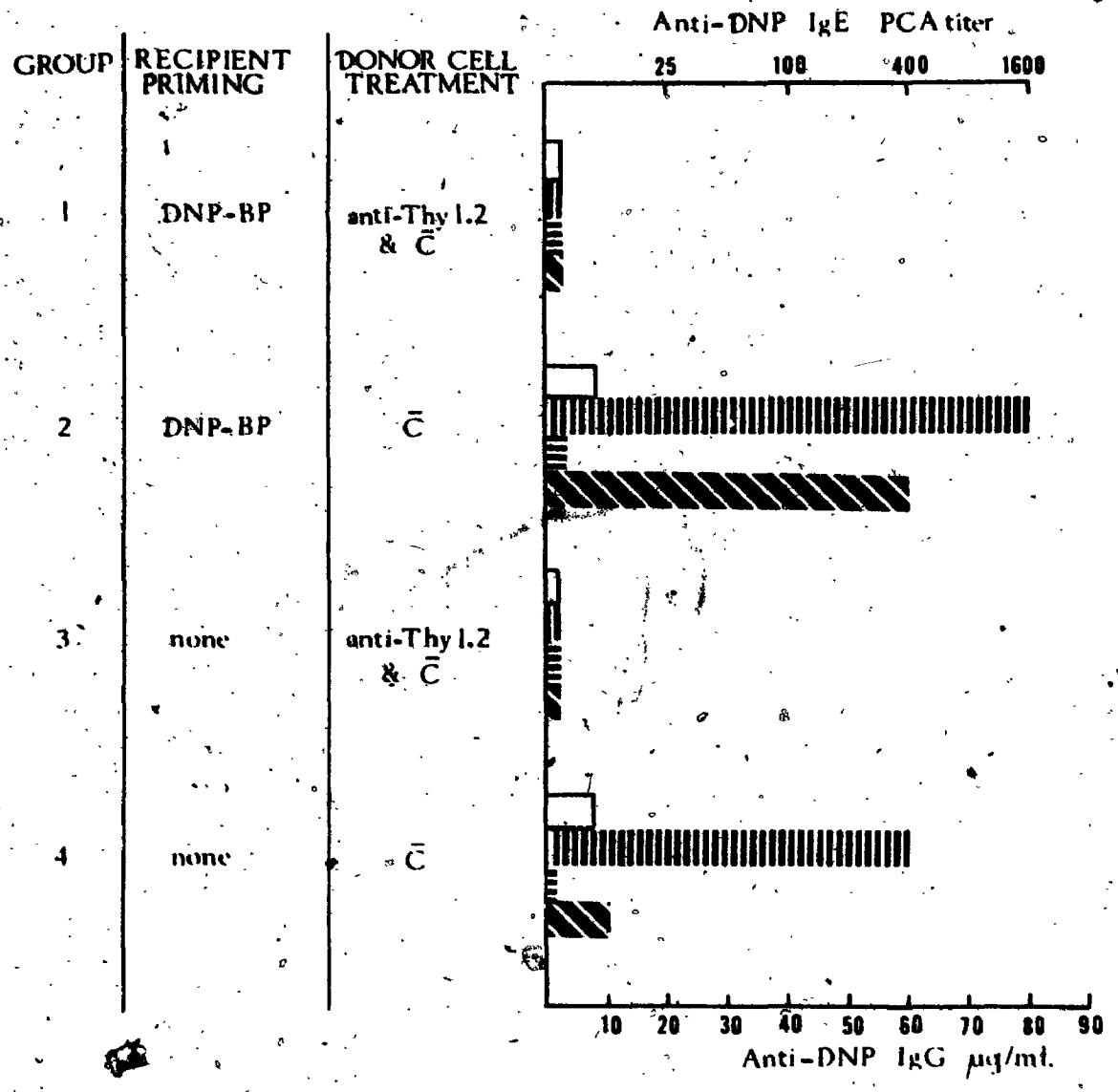
4.5 Absence of Hapten-reactive T Helper Cells for IgE in DNP-BP-Treated Mice:

We have shown earlier that priming of CBA mice with DNP-BP resulted in an accelerated appearance of anti-DNP IgE and IgG antibodies following subsequent immunization with DNP-OA alum (Fig. 4). It suggested 2 alternative possibilities, (i) the generation of hapten-reactive T helper cells, or (ii) the induction of anti-DNP IgE B-memory cells by DNP-BP treatment. These possibilities were evaluated by adoptive transfer experiments.

Donor mice were primed with 10^9 DNP-BP a month before adoptive transfer. Recipient mice were primed with 10^9 DNP-BP one week prior to adoptive transfer or were left unprimed. On the day of transfer all recipients were lethally irradiated (960 R), and injected i.v. with 5×10^7 donor spleen cells previously treated with anti-Thy 1.2 and complement, or complement alone. The recipients were then immunized with 2 μg DNP-OA in alum. Mice were bled and anti-DNP IgE and IgG levels were determined. Results are shown in Fig. 5. It can be seen that mice reconstituted with anti-Thy 1.2 and complement-treated donor spleen cells (T cell-depleted spleen cell population) failed to produce any detectable levels of anti-DNP antibodies upon challenge with DNP-OA (groups 1 and 3). On the other hand, mice which received donor spleen cells treated with complement alone, mounted both anti-DNP IgE and IgG responses following DNP-OA injection (groups 2 and 4).

These results indicate that DNP-BP primed recipients like, unprimed recipients, when exposed to lethal irradiation and reconstituted with T cell-depleted donor spleen cells were unable to mount anti-DNP responses upon challenge with DNP-OA. Therefore, both groups of recipients were equally deficient in providing T cell help to donor B cells for antibody production. Upon reconstitution with complement-treated donor spleen cells both DNP-BP primed and non-primed recipients produced anti-DNP IgE and IgG antibodies. In this case the magnitude of the responses was significantly higher in primed recipients than in the unprimed ones (groups 2 and 4).

Figure 5. Absence of hapten-reactive T helper cells in DNP-BP-primed mice. Donor mice were primed with 10^9 DNP-BP a month before adoptive transfer. Their spleen cells were either treated with anti-Thy 1.2 and complement, complement alone or were left untreated. Recipient mice were primed with 10^9 DNP-BP one week prior to the cell transfer. They were lethally irradiated (960 R) on the day of adoptive transfer and received 5×10^7 donor spleen cells. Two hrs. following cell transfer mice were challenged with 2 µg DNP-OA alum. Anti-DNP IgE antibodies are shown on days 7 () and 10 () and anti-DNP IgG on days 7 () and 10 ().


GROUP
RECIPIENT PRIMING
DONOR CELL TREATMENT
1
DNP-BP
anti-Thy 1.2 & C̄
2
DNP-BP
C̄
3
none
anti-Thy 1.2 & C̄
4
none
C̄
Anti-DNP IgE PCA titer
25
100
400
1600
10
20
30
40
50
60
70
80
90
Anti-DNP IgG μg/ml.

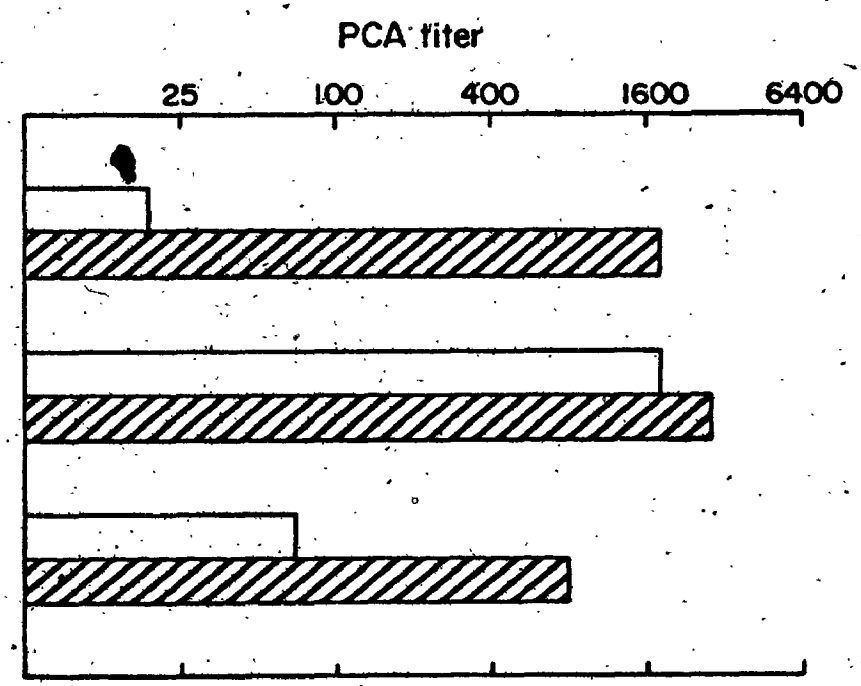
Taken collectively these results ruled out the presence of hapten-reactive T helper cells in DNP-BP primed mice but suggested the appearance of radio-resistant IgE B-memory cells.

4.6 Demonstration of Hapten-specific IgE B-memory Cells in the Spleens of DNP-BP Primed Mice by an Adoptive Transfer:

The previous findings strongly suggested the presence of IgE B-memory cells in DNP-BP primed mice (Fig. 4 and 5). To demonstrate this, groups of donor mice were primed with 10^9 DNP-BP one month prior to adoptive transfer (these provided the source of IgE B-memory cells). Another group was immunized with 0.5 µg OA in alum one week before adoptive transfer (the source of carrier primed T helper cells). On day 0, syngeneic recipient mice were x-irradiated (660 R) and reconstituted with 2 types of donor spleen cells (2×10^7 from each) in different combinations. Following cell transfer recipient mice were immunized with 2 µg DNP-OA in alum and were bled 7 and 10 days later. The results presented in Fig. 6 show that transfer of spleen cells from DNP-BP primed mice along with cells from OA primed mice gave the characteristic secondary anti-DNP IgE response (day 7 PCA titer of 1:2000), while the co-transfer of normal spleen cells along with cells from OA-primed donors resulted on day 7, in a PCA titer of only 1:80. The group which received DNP-BP primed spleen cells along with normal spleen cells, developed on day 7 poor IgE response (PCA titer 1:20) but on day 10 when the normal spleen cells had the opportunity to acquire OA-primed T helper cells the response reached levels as comparable to those in the group receiving OA and DNP-BP primed spleen cells. These results clearly show that

Figure 6. Demonstration of hapten specific IgE B-memory cells in the spleens of DNP-BP-treated mice. Donor mice were immunized with 0.5 μg OA in alum one week before, or with 10^9 DNP-BP, 4 weeks prior to the cell transfer. On the day of transfer 2×10^7 spleen cells from each type of donor were mixed and transferred to x-irradiated (660 R) syngeneic recipients. The recipients were challenged with 2 μg DNP-OA alum on the same day. ☐ Day 7 anti-DNP PCA titer. ▨ Day 10 anti-DNP PCA titer.

CELLS TRANSFERRED	
Donor 1	Donor 2
Normal	DNP-BP
OA	DNP-BP
OA	NORMAL



immunization with DNP-BP stimulates IgE B-memory cells in CBA mice and that spleen cells from DNP-BP primed mice were capable of mounting an anti-DNP IgE response, after being removed from the DNP-BP treated host and allowed to collaborate with the appropriate carrier-primed T helper cells.

Taken together the preceeding results suggested that a suppressive environment selective for IgE is generated in mice, treated with DNP-BP and that it is responsible for the inability of IgE B-memory cells to convert into IgE secreting plasma cells.

4.7 Search for IgE Selective Suppressor Cells in the Spleens of DNP-BP Treated Mice:

The inability of CBA (and other non-responder strains) to produce IgE antibodies following immunization with DNP-BP (Fig. 3), coupled with the sharp drop in anti-DNP IgE levels following treatment with DNP-BP and booster with DNP-OA in CBA mice (Fig. 4) suggested that treatment with DNP-BP generates a suppressive mechanism(s), which might operate via T suppressor cells. To evaluate this possibility, the following experiments were carried out.

(a) Sensitivity to X-icradiation and Cyclophosphamide Treatment:

The presence of antigen nonspecific, IgE selective, T suppressor cells in nonresponder mice and their sensitivity to low dose x-irradiation and CY treatment has been shown previously by Watanabe et al. (1976) and Chiorazzi et al., (1976, 1977). In order to eliminate such putative suppressor cells separate groups of CBA mice were subjected to a low dose of x-irradiation (250 R), or were treated

TABLE IV

Effect of pretreatment with low dose x-irradiation or cyclophosphamide on the anti-DNP-BP responses of CBA/J mice.

Group	Treatment	Anti-DNP IgE (PCA titer)[c] on days 14	28	35	42
1	X-R[a] (250 R) (day 0)	<10	<10	200	40
2	CY[b] (60 mg/kg) (day -3)	10	10	20	10
3	CY[b] (150 mg/kg) (day -3)	<10	<10	100	20
4	None	<10	<10	20	10

[a] Mice were exposed to x-irradiation 2-3 hrs. prior to the immunization.

[b] Cyclophosphamide base was injected i.p. 3 days prior to the immunization.

[c] Mice were immunized on day 0 and were boosted on day 28 with 10^9 DNP-BP in alum. Anti-DNP IgE titers were determined on days shown.

with either 60 mg or 150 mg/kg body weight cyclophosphamide (CY), prior to primary immunization with DNP-BP. All mice were primed with 10^9 DNP-BP in alum on day 0 and were boosted on day 28. Table IV shows that regardless of whether mice were pretreated or not, the pattern of unresponsiveness for IgE production remained the same during the primary response (day 14 and day 28 PCA titers). However, following secondary immunization, mice exposed to 250 R (group 1), or treated with 150 mg/kg CY, prior to the primary immunization (group 3), mounted good secondary anti-DNP IgE response. The IgE PCA titers of groups 1 and 3 were 10 and 5 fold higher respectively than those determined for the untreated group (group 4). However, these titers were short-lived and decreased 5 fold within a week.

These results indicated that pretreatment of mice with low dose irradiation or 150 mg CY did increase to some extent their responsiveness to DNP-BP following booster, but the primary response was unaffected.

When CBA mice were exposed to low dose x-irradiation or to CY treatment 3 days before booster (day 25), the secondary anti-DNP IgE response remained unchanged from the nontreated control group (results not shown).

(b) Demonstration of Suppression by Adoptive Transfer:

To determine the presence of putative, IgE selective, T suppressor cells groups of donor mice were injected with 10^9 DNP-BP once (on day -14), or to enhance the suppressive activity, 3 times (on day -16, -14, -12 relative to the day of adoptive transfer). On the day of transfer normal syngeneic, recipient mice received donor spleen cells and were challenged with 2 µg DNP-OA in alum. The day 14

and 21 anti-DNP IgE and IgG responses are presented in Table V. Each IgE and IgG value in group 1 and 3 represents a geometric mean of 6 experiments performed under identical conditions. As the experiments were not performed at the same time, they could not be considered replicates for statistical analysis. The data were therefore, subjected to an analysis of variance without replication. It can be seen that sera of recipients of spleen cells from normal or DNP-BP treated donors gave similar PCA titers on day 14 (1:466 vs 1:400 vs 1:329). On day 21, however, a small but statistically significant difference between group 1 and 3 was recorded (1:306 vs 1:131, $p < 0.01$). The statistical significance of the difference in PCA titers between groups 1 and 2 could not be evaluated since the adoptive transfer with group 2 donor cells was performed only once. Anti-DNP IgG levels were not suppressed in the recipients of DNP-BP spleen cells, and the increases observed were not statistically significant. Anti-OA PCA titers ranged between 1:100 to 1:400 and no significant differences among the groups were observed (not shown). When recipients of spleen cells were challenged with 2 μg OA instead of DNP-OA, the anti-OA IgE levels were similar to those of recipients of normal spleen cells (not shown), indicating that DNP-BP primed spleen cells did not interfere in the induction of a response against an irrelevent antigen. Thus it appears that spleen cells from DNP-BP primed donors were capable of transferring weak but detectable suppression of the anti-DNP IgE response. The suppression was selective for the IgE class, appeared always late (day 21) and acted in an antigen-specific manner.

TABLE V

Adoptive transfer of spleen cells from DNP-BP-treated mice to normal recipients.

Group	Donor treatment[a]	Anti-DNP antibodies IgE (PCA titer)[b] on day 14	on day 21	IgG (µg/ml)[c] on day 14	on day 21
1	None	466	306	33	57
2	DNP-BP x 1[e]	400	160	42	68
3	DNP-BP x 3	329	131[d]	79	71

[a] Donor mice were left untreated or were injected with 10^9 DNP-BP on day -14 (x 1) or days -16, -14, and -12 (x 3) before transfer. On day 0, groups of 4 normal recipients were injected with 6×10^7 donor spleen cells and were boosted with 2 µg DNP-OA in alum.

[b] Expressed as the geometric mean of PCA titers of six separate experiments, for groups 1 and 3.

[c] Geometric means of six separate experiments expressed in µg/ml serum, for groups 1 and 3.

[d] Statistically significant difference from control on the same day $p < 0.01$.

[e] No replicate experiments in this group.

(c) DNP-BP Induced Suppression is not Mediated by T Cells:

In order to determine whether the weak and delayed suppression observed upon transfer of DNP-BP-treated spleen cells to normal recipients could be enhanced after removal of the DNP-primed B cells from the donor spleens, donor mice were injected with 10^9 DNP-BP 16, 14 and 12 days prior to adoptive transfer. On the day of transfer donor spleen cells were incubated on anti-mouse Ig-coated dishes and 2×10^7 purified splenic T cells were transferred to normal syngeneic recipients which were then challenged with 2 µg DNP-OA in alum. Anti-DNP antibody levels were determined on days 14, 21 and 28 following cell transfer. Figure 7 shows that the recipients of pure T cells obtained from either DNP-BP-treated donors or from normal donors gave similar anti-DNP IgE responses. The mice which received unseparated spleen cells from DNP-BP-treated donors (group 3) showed a PCA titer of 1:100 on day 14 which subsequently decreased to 1:40 on day 21. This group had significantly lower PCA titers than the group receiving unseparated spleen cells from normal donors (PCA titers of 1:40 vs 1:200 on both day 21 and 28).

These data clearly show that splenic T cells from DNP-BP-treated mice were not involved in IgE-selective suppression. These findings, however, were confirmed by an adoptive transfer experiment in which spleen cells from DNP-BP-treated donors were treated with monoclonal anti-Thy 1.2 antibodies and complement, prior to transfer. Table VI shows that the transfer of 5×10^7 anti-Thy 1.2 and complement treated spleen cells to normal recipients did not remove the ability of these cells to provide the day 21 suppression observed in previous experiments where intact spleen cells were transferred (Table V).

Figure 7. DNP-BP induced suppression is not mediated by splenic T cells. Donor spleen cells were incubated on rabbit anti-mouse IgG (RAMG) coated dishes (3×10^7/plate) and nonadherent cells were transferred into normal syngeneic recipients (2×10^7/recipient). In separate groups 5×10^7 unseparated spleen cells from the same donors were transferred and all mice were challenged with 2 µg DNP-OA alum 2 hrs. following cell transfer. Anti-DNP IgE levels were detected 14 (▥), 21 (▨) and 28 (□) days after challenge.

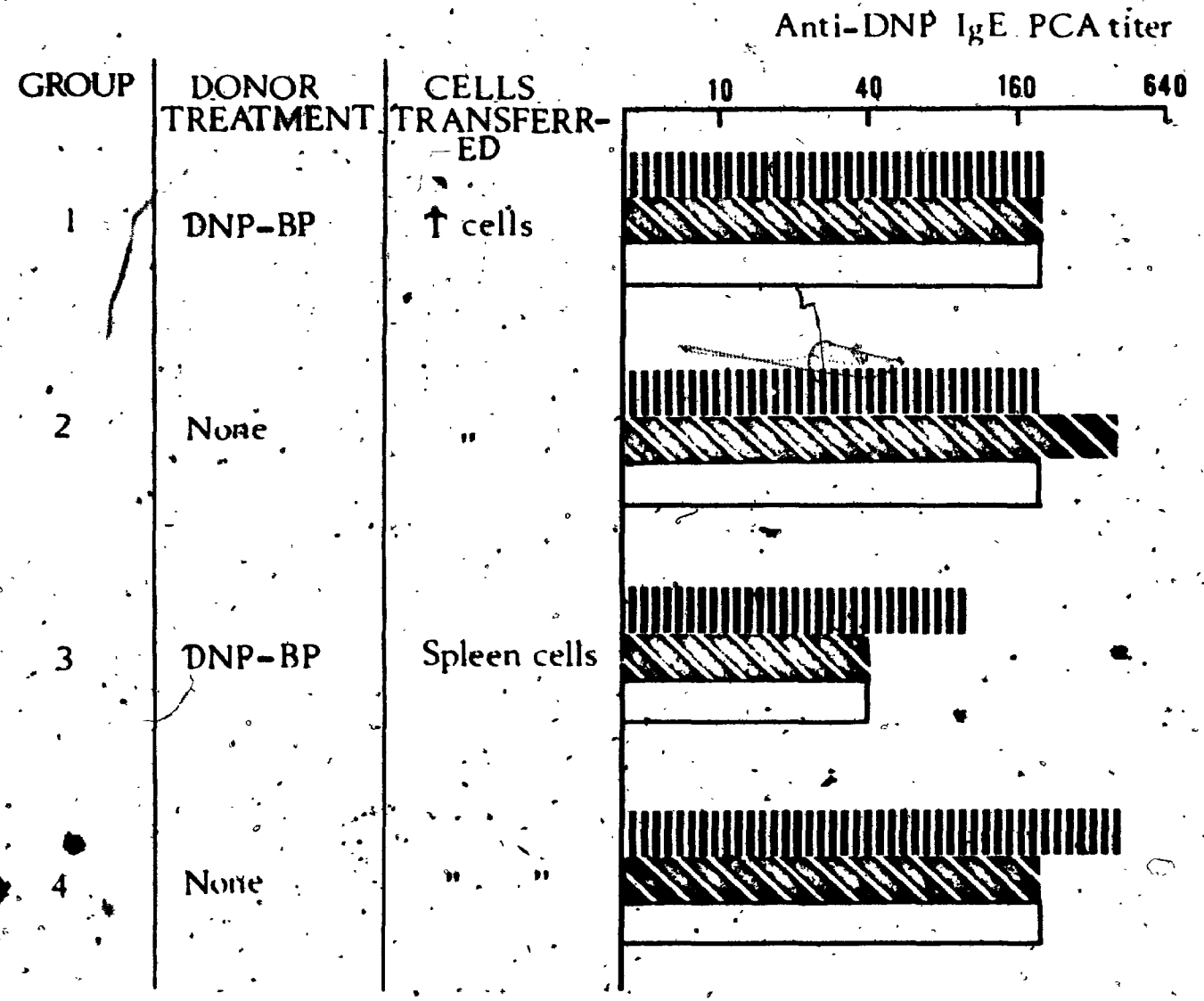

Anti-DNP IgE PCA titer
GROUP
DONOR TREATMENT
CELLS TRANSFERR-ED
10
40
160
640
1
DNP-BP
cells
2
None
"
3
DNP-BP
Spleen cells
4
None
"
"

TABLE VI

Transfer of spleen cells after treatment with anti-Thy 1.2 serum and complement.

Group	Treatment of donor spleen cells[a]	Anti-DNP Antibodies IgE (PCA) on day		IgG (μg/ml) on day	
		14	21	14	21
1	Anti-Thy 1.2 + complement	400	100	56	71
2	Complement	400	80	57	46

[a] Donor mice were injected i.p. three times with 10^9 DNP-BP (day -16, -14, and -12). On day 0 their spleen cells were treated with anti-Thy 1.2 and complement or with complement alone. Normal syngeneic recipient mice were injected with 5×10^7 donor spleen cells (cells were counted before treatment as 5×10^7/recipient) and were immunized with 2 μg DNP-OA in alum 2 hr. after cell transfer.

Moreover, there was no essential difference between PCA titers of the recipients of anti-Thy 1.2 plus complement treated cells and the complement treated controls. These data clearly show that IgE selective suppression observed in DNP-BP-treated, DNP-OA (alum) challenged mice is not mediated by splenic T suppressor cells; alternatively their suppressive activity could have been lost during the transfer manipulations.

4.8 Transfer of Primed Spleen Cells into DNP-BP-treated Recipients:

The discrepancy between the strong suppression observed *in situ* in DNP-BP-immunized mice (Figs. 1, 3 and 4) and the weak and delayed suppression operating in adoptive transfer of DNP-BP spleen cells to normal recipients (Table V) was intriguing and raised the possibility that the cells involved had lost much of their ability to confer suppression, once removed from their original host. In order to test this assumption, donor mice were immunized with 2 μg OA (alum) a month prior to cell transfer. Recipient mice were either treated with 10^9 DNP-BP, with 2 μg TNP-KLH or with saline (control), 2 weeks before cell transfer. On the day of adoptive transfer, $5 * 10^7$ donor spleen cells/recipient were transferred and the recipients were challenged with 2 μg DNP-OA in alum. This protocol had the advantage of allowing assessment of the DNP-BP environment on the DNP-primed B cells *in situ*, when provided with the appropriate carrier primed T helper cell (from the OA primed donor). Anti-DNP IgE and IgG responses were determined 7 and 14 days following cell transfer. Results are shown in Table VII. The recipients treated with DNP-BP (group 1) showed anti-DNP IgE PCA titer of 1:200 on day 7 which decreased 10 fold within a week (day 14 PCA titer 1:20). It is

TABLE VII

Suppression of OA primed spleen cells in DNP-BP-treated recipients upon challenge with DNP-OA

Group	Recipient's treatment	Anti-DNP Antibodies IgE (PAC titer) on day		IgG (µg/ml) on day	
		7	14	7	14
1	10^9 DNP-BP x 3 (day -16, -14, -12)	200	10	344	474
2	2 µg TNP-KLH x 3 (day -16, -14, -12)	800	200	57	155
3	None	200	100	11	24

Donor mice were primed with 2 µg OA in alum one month prior to the adoptive transfer. Recipient mice were either nontreated or treated with 10^9 DNP-BP or 2 µg TNP-KLH 3 times i.p. On day 0 donor spleen cells were injected i.v. (5×10^7 cells/recipient) and then immunized with 2 µg DNP-OA (A1) 2 hr. after cell transfer.

obvious that recipients which were treated with TNP-KLH or with saline elicited on day 14 PCA titers of 1:200 and 1:100 respectively. This represents a 10-20 fold increase over the DNP-BP-treated group.

It, therefore, appeared that mice treated with DNP-BP provided an environment condusive to the development of a considerable degree of suppression of the IgE antibody response.

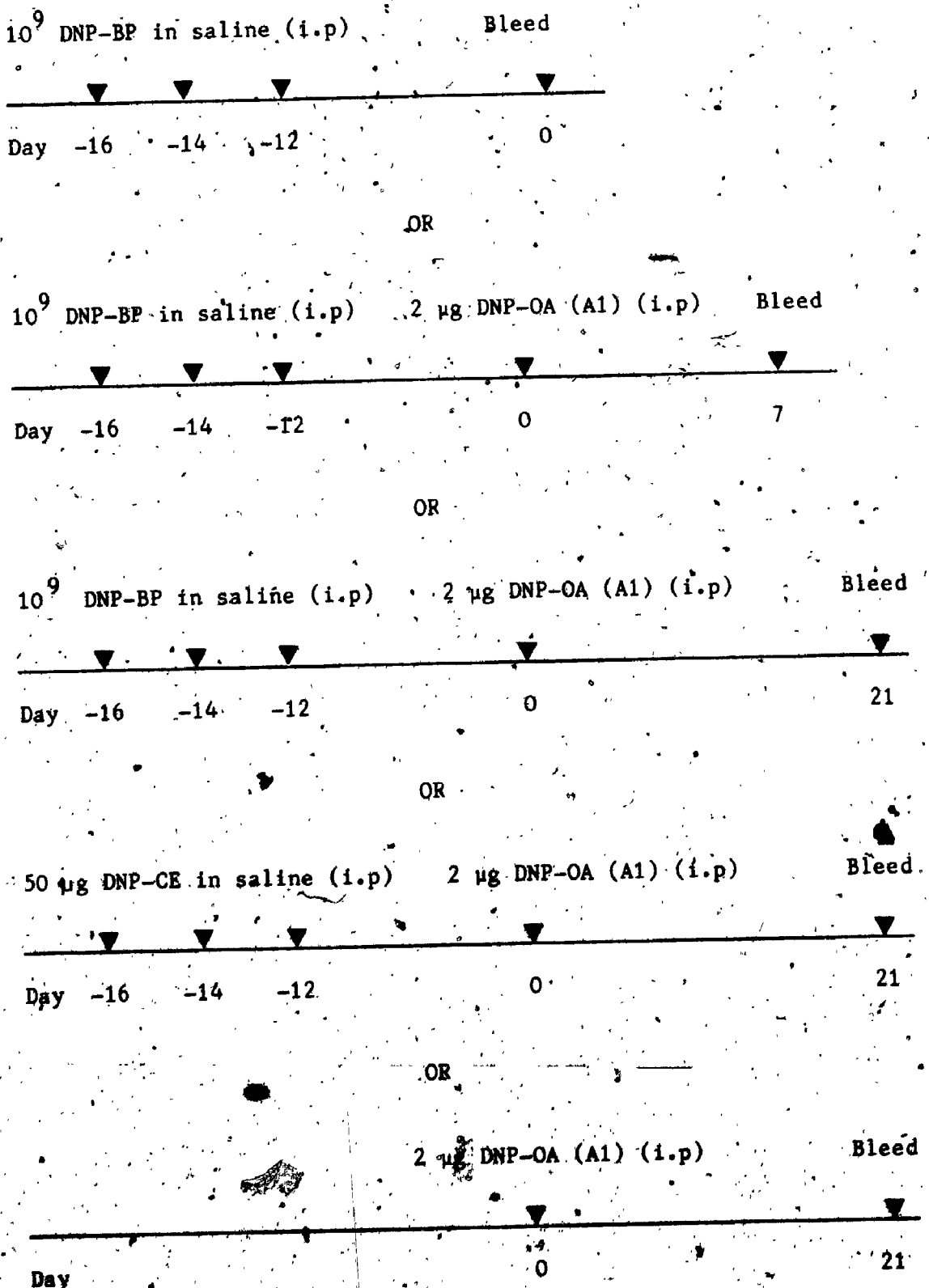
4.9 Suppression of Anti-DNP IgE Antibody Response by Passive Transfer of Serum:

We have shown that pretreatment of mice with DNP-BP resulted in IgE selective suppression following immunization with DNP-OA (Fig. 4). Adoptive transfer of OA-primed spleen cells in DNP-BP-treated mice also resulted in IgE-selective suppression upon challenge with DNP-OA (Table VII). However, the suppressive activity was only marginal upon transfer of spleen cells or T-depeleted spleen cells from DNP-BP-treated donors to normal recipients (Table V and VI). These results suggestd the possibility that the suppressive activity might be transferred more effectively through serum rather than spleen cells. The following experiments, therefore, focused on the serum obtained from these animals to understand the mechanism(s) of IgE selective suppression operating in DNP-BP-treated mice.

(a) Suppressive Effect of the Serum On the Induction of IgE Response:

Sera for passive transfer were obtained as illustrated in Fig. 8. Briefly, mice treated 3 times (day -16, -14, -12) with 10^9 DNP-BP in saline, i.p. were bled on day 0 or were challenged with 2 μg DNP-OA in alum on day 0 and then were bled either 7 or 21 days following

Figure 8. Preparation of sera for passive transfer. The sera were used nonabsorbed (NAS) or absorbed (AS) on DNP-BSA- and OA-sepharose immunosorbents.


10^9 DNP-BP in saline (i.p)
Bleed
Day -16 -14 -12 0
OR
10^9 DNP-BP in saline (i.p) 2 µg DNP-OA (A1) (i.p) Bleed
Day -16 -14 -12 0 7
OR
10^9 DNP-BP in saline (i.p) 2 µg DNP-OA (A1) (i.p) Bleed
Day -16 -14 -12 0 21
OR
50 µg DNP-CE in saline (i.p) 2 µg DNP-OA (A1) (i.p) Bleed
Day -16 -14 -12 0 21
OR
2 µg DNP-OA (A1) (i.p) Bleed
Day 0 21

DNP-OA injection. Mice treated similarly with 50 µg DNP-CE or untreated were also immunized with 2 µg DNP-OA alum on day 0 and were bled 21 days later. The serum obtained from normal mice served as an additional control. Aliquots of sera obtained from mice treated with DNP-BP or DNP-CE and then challenged with DNP-OA were absorbed exhaustively with DNP-BSA-Sepharose and OA-Sepharose immunosorbents to remove anti-DNP and anti-OA antibodies. Similar aliquots of sera were stored unabsorbed at -20°C until needed. Complete removal of antibodies was assessed by RIA.

Absorbed (AS) or nonabsorbed (NAS) sera from each donor group were injected into a separate group of recipients. Each animal received 2 injections (0.25 ml each) of serum, i.v. at 24 hr. intervals. Twelve hrs. after the first serum injection the recipients were immunized with 2 µg DNP-OA in alum and were boosted 28 days later. Anti-DNP IgE and IgG levels were determined 14, 28 and 35 days following primary immunization. Results shown in Table VIII indicated that nonabsorbed serum (NAS) from DNP-BP-treated mice without DNP-OA challenge, failed to show any suppressive effect on anti-DNP IgE response (days 14 and 28 IgE titers 1:640 and 400 respectively), while the NAS from DNP-BP-treated, DNP-OA injected mice had remarkable suppressive effects on the induction of primary anti-DNP IgE response (PCA titer 1:20 on day 14). The same serum after removal of anti-DNP and anti-OA antibodies remained suppressive for the induction of anti-DNP IgE antibodies (group 4, day 14 and 28 PCA titers 1:113 and 1:80 respectively). The AS obtained from DNP-BP-treated mice 7 days after DNP-OA injection also showed similar suppressive effects. On the other hand AS obtained from DNP-CE-

[a] Normal syngeneic mice were injected twice, i.v., 0.25 ml/injection, at 24 hr. interval. Twelve hrs. following the first serum injection (day 0) recipients were immunized with 2 μg DNP-OA in alum and were boosted 28 days later.

[b] Donors were bled on day 0.

[c] Donors were bled 7 days after DNP-OA injection.

[d] Donors were bled 21 days after DNP-OA injection.

[e] Results are expressed as geometric mean of PCA titers of 3 separate experiments.

[f] Geometric means of IgG values of 3 separate experiments.

[g] Not determined.

TABLE VIII

Suppression of anti-DNP IgE response by passive transfer of absorbed (AS) and nonabsorbed (NAS) serum.

Group	Donor Treatment (i.p.)	Serum Treatment	Anti-DNP Antibodies[a] IgE (PCA titer) on day 14	28	35	IgE (μg/ml) on day 14	35
1	10^9 DNP-BP x 3 (day -16,-14, -12)[b]	None	640	400	ND[g]	ND	ND
2	10^9 DNP-BP x 3 (day -16,-14, -12)[d] 2 µg DNP-OA (A1) (day 0)	"	20	10	1000	20	109
3	"[c]	Absorption with DNP-BSA-sepharose and OA-sepharose	160	80	800	19	334
4	"[d]	"	113[e]	80[e]	800[e]	26[f]	197[f]
5	50 µg DNP-CE x 3 (day -16,-14, -12) 2 µg DNP-OA (A1) (day 0)[d]	"	800	400	ND	37	ND
6	2 µg DNP-OA (A1) (day 0)[d]	None	800	400	2000	47	317
7	NMS	"	1280[e]	400[e]	2530[e]	24[f]	365[f]
8	Nil	Nil	800	400	2000	35	358

treated mice, NAS from nontreated but DNP-OA injected mice or serum from normal mice showed the expected unsuppressed PCA titers after primary immunization. However, the secondary anti-DNP IgE responses were comparable in all groups of recipients and were not suppressed. The primary and secondary anti-DNP IgG responses were again comparable in all groups except group 2, where secondary anti-DNP IgG levels were lower than in all the other groups (day 35, 109 µg IgG/ml).

The data presented in Table VIII ruled out the possibility of antibody-feedback as a mechanism for suppression in groups receiving absorbed serum. Furthermore, these results suggest that AS (from DNP-BP-treated mice) contains powerful suppressor molecules which are selective for the IgE antibody response.

(b) Suppressive Effect of Absorbed Serum (AS) on an Ongoing IgE Response

The AS obtained from DNP-BP-treated mice showed significant suppressive effect on the induction of anti-DNP IgE antibody response (Table VIII). The question arose as to whether this absorbed serum could interfere with an ongoing IgE antibody response. In an attempt to answer this question, recipient mice were immunized with 2 µg DNP-OA in alum and 10 days later were injected i.v. with 0.5 ml of AS obtained from DNP-BP-treated mice. A group of DNP-OA primed mice was injected with 0.5 ml of NMS and served as control. The anti-DNP IgE and IgG levels were determined 14, 21 and 28 days following DNP-OA immunization. Results are shown in Fig. 9. Recipients of AS obtained from DNP-BP-treated mice did not show any significant difference in PCA titers on day 14 (3 days after serum transfer) from those

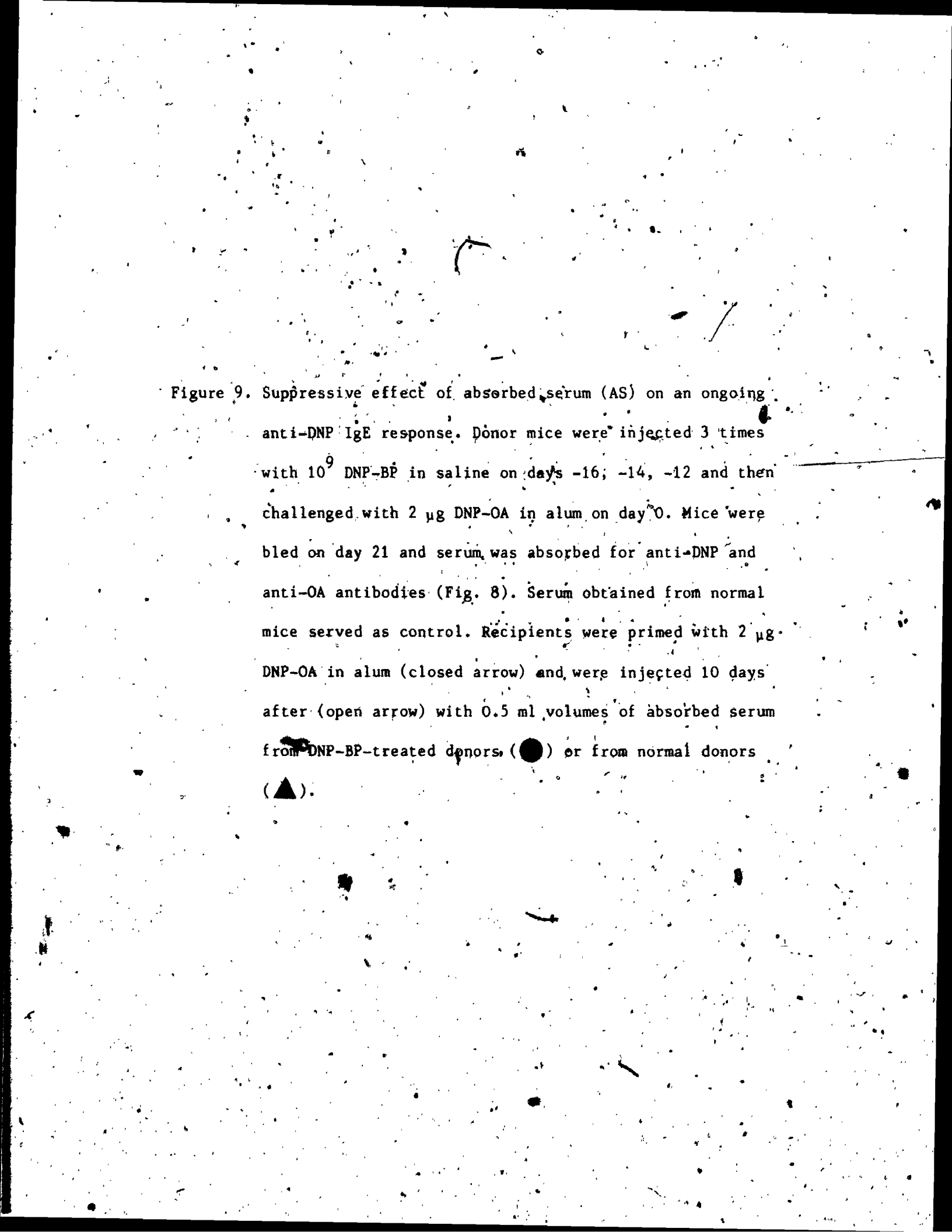
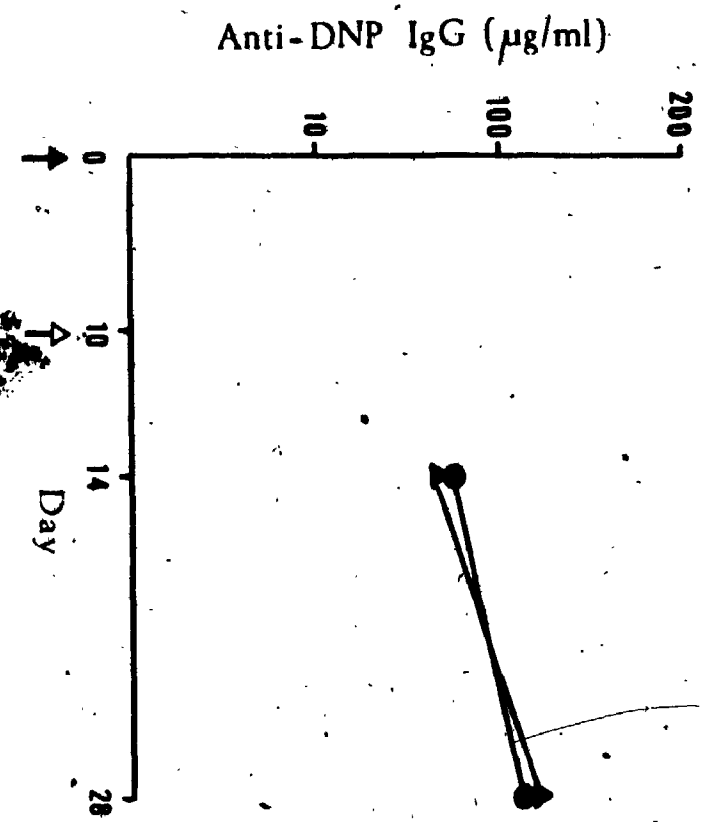


Figure 9. Suppressive effect of absorbed serum (AS) on an ongoing anti-DNP IgE response. Donor mice were injected 3 times with 10^9 DNP-BP in saline on days -16, -14, -12 and then challenged with 2 μg DNP-OA in alum on day 0. Mice were bled on day 21 and serum was absorbed for anti-DNP and anti-OA antibodies (Fig. 8). Serum obtained from normal mice served as control. Recipients were primed with 2 μg DNP-OA in alum (closed arrow) and were injected 10 days after (open arrow) with 0.5 ml volumes of absorbed serum from DNP-BP-treated donors (●) or from normal donors (▲).


Anti-DNP IgE (PCA Titer)
100
200
400
800
0
10
14
21
28
Day


Anti-DNP IgG (μg/ml)
10
100
200
0
10
14
28
Day

injected with NMS. However, days 21 and 28 PCA titers (of the group which received AS from DNP-BP-treated mice) were significantly lower as compared to the control group (PCA titer 1:100 vs 1:400 and 1:80 vs 1:400 respectively). The anti-DNP IgG levels were comparable in groups injected with either serum and were not suppressed.

These results indicated that AS obtained from DNP-BP-treated mice could also suppress an ongoing IgE anti-DNP response but this suppression was less remarkable than the one obtained where AS was allowed to interfere with the induction phase of the response.

(c) Effect of In Vitro Treatment of Normal Spleen Cells with AS:

Studies done thus far revealed that the suppressive activity present in AS obtained from DNP-BP-treated mice, not only interfered with the induction of an antibody response upon passive transfer into normal mice (Table VIII) but also impaired an ongoing IgE response in DNP-OA-primed mice (Fig. 9). The question as to whether the putative suppressor molecule(s) present in the serum of DNP-BP-treated mice would also be effective in vitro was addressed in the following experiment.

Spleen cells from normal donors were incubated in vitro for 18 hrs. in RPMI-1640 medium containing either 6% of the AS from DNP-BP-treated mice, or with an equivalent concentration of NMS (see 'Materials and Methods' section 3.19). The cells were harvested, washed twice with RPMI-1640, suspended in the same medium and 1.5×10^7 viable cells/recipient were transferred i.v. into sub-lethally irradiated (660 R) syngeneic mice. All mice were then immunized with 2 µg DNP-OA in alum on the same day and were

TABLE IX

Effect of *in vitro* treatment of normal spleen cells with absorbed serum (AS)

Group	Serum used in culture[a]	Anti-DNP Antibodies IgE (PCA titer) on day			IgG (μ g/ml) on day		
		14	21	35	14	21	35
1	AS[b]	20	40	4000	<1	<1	32
2	NMS[c]	640	400	2000	<1	<1	125

[a] Normal spleen cells at a concentration of 10^7/ml were treated *in vitro* for 18 hrs. in RPMI-1640 supplemented with 6% serum from DNP-BP-treated or from normal mice.

[b] Serum was obtained on day 21 after treatment with 10^9 DNP-BP on days -16, -14, -12 and priming with 2 μg DNP-OA in alum on day 0. The serum was absorbed on DNP-BSA-sepharose and OA-sepharose.

[c] Normal mouse serum.

boosted 28 days later. The results are shown in Table IX. The mice reconstituted with spleen cells, treated with AS containing suppressive activity, showed highly suppressed anti-DNP IgE responses on days 14 (PCA titer of 1:20 vs 1:640 in control), and 21 (1:40 vs 1:400); however, following booster, the secondary IgE response was not suppressed (PCA titer 1:4000 on day 35). The primary anti-DNP IgG responses were undetectable in both groups. The secondary anti-DNP IgG response was lower in group 1 then group 2 (control).

These results indicated that in vitro treatment of normal spleen cells with AS from DNP-BP-treated mice affects their ability to mount a primary anti-DNP IgE antibody response and suggests, therefore, that the putative suppressor molecule(s) adsorbs to cell targets present in normal mouse spleen.

(d) The Suppressive Activity Present in the Serum of DNP-BP-Treated CBA/J Mice Operates Across MHC Barrier:

Sera from CBA ($H\text{-}2^k$) mice treated with DNP-BP and primed with DNP-OA alum, obtained 21 days after priming, were absorbed as described earlier (Table VIII, Fig. 9) and 0.25 ml were injected i.v. to DBA/2J ($H\text{-}2^d$) mice twice, at 24 hr. intervals. Other groups were treated with normal mouse (CBA) serum or were left untreated. All mice were primed with 2 µg DNP-OA in alum 12 hrs. after the first serum injection. The results are shown in Table X. It can be seen that mice injected with serum obtained from DNP-BP treated donors had a marked suppressive effect on the induction of both anti-DNP IgE and IgG responses. A PCA titer of 1:10 was detected 14 days after DNP-OA

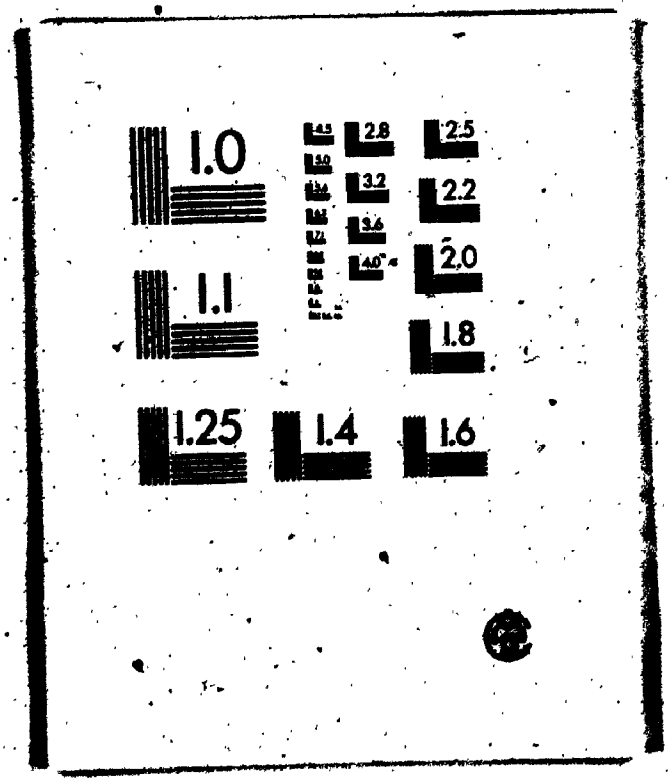


TABLE X

Suppressive activity of the absorbed serum (AS) obtained from DNP-BP-treated CBA/J mice across MHC barrier.

Group	Recipient treatment[a]	Anti-DNP Antibodies IgE (PCA titer) on day 14	IgE (PCA titer) on day 28	IgG (μ g/ml) on day 14	IgG (μ g/ml) on day 28
1	AS from DNP-BP-treated mice[b]	10	<10	5	2
2	NMS[c]	320	320	43	62
3	None	320	200	38	44

[a] DBA/2J ($H\text{-}2^d$) mice were injected twice with 0.25 ml volumes of CBA/J serum at 24 hr interval. Priming was with 2 μg DNP-OA in alum 12 hr after the first injection of serum.

[b] Day 21 serum was obtained from CBA mice treated with 10^9 DNP-BP on days -16, -14, -12 and immunized with 2 μg DNP-OA in alum on day 0. The serum was absorbed on DNP-BSA-sepharose and OA-sepharose.

[c] Normal mouse (CBA) serum.

immunization and on day 28 the response was completely abolished. The administration of serum obtained from normal CBA/J mice, however, did not affect the anti-DNP antibody responses.

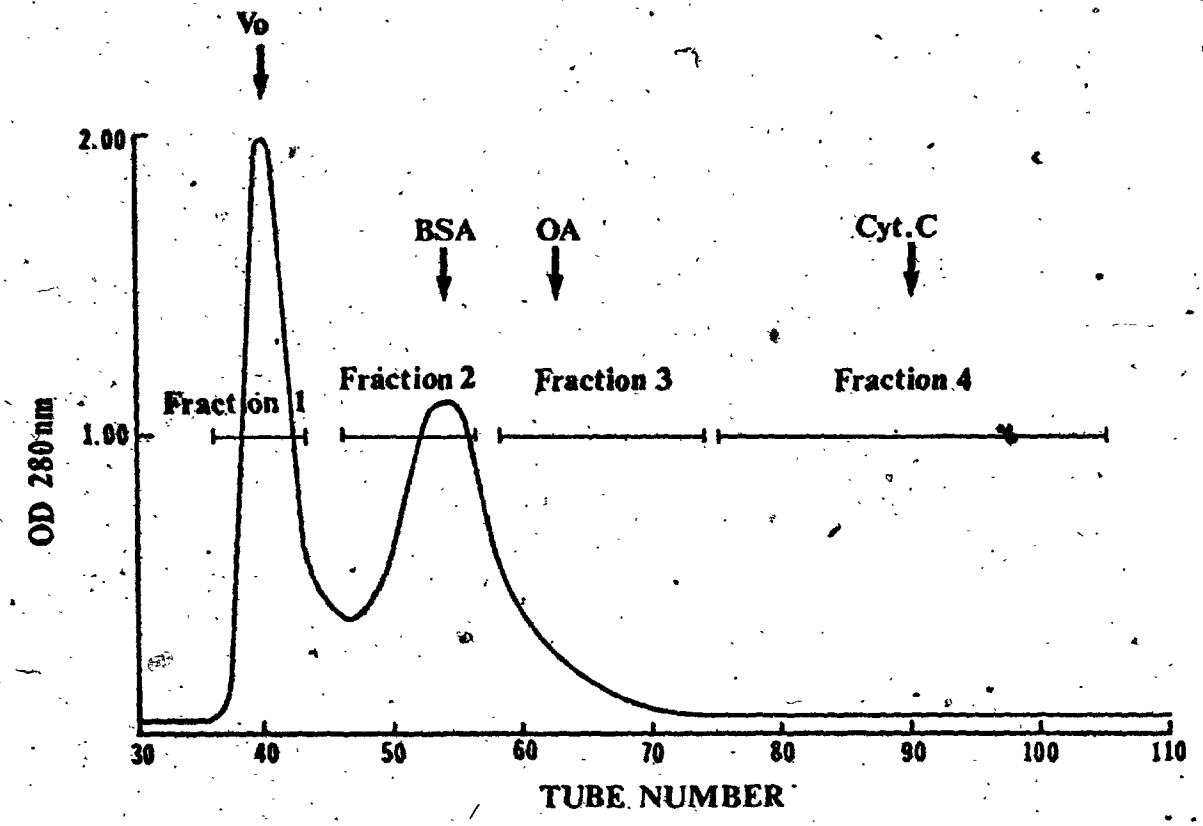
These results clearly demonstrated that the suppressive activity present in the AS obtained from DNP-BP-treated mice acts across the MHC barrier.

4.10 Isolation of the Suppressive Activity of Serum by Gel Filtration:

In the preceeding experiments we established that sera of CBA mice treated with DNP-BP and immunized with DNP-OA in alum, suppressed very effectively the IgE antibody response in a hapten-specific and isotype-dominant fashion. The suppression operated both in situ, upon passive transfer of serum to normal, (Table VIII) and DNP-OA primed recipients (Fig. 9), or in vitro following incubation with normal syngeneic spleen cells (Table IX).

The important element of this suppression is its high effectiveness, even after removal of anti-DNP and anti-OA antibodies from the serum. It was obvious, therefore, that the suppression observed was dependent upon a mechanism other than antibody-feedback and operated through suppressor molecules that failed to bind to the antigen. In order to attempt to isolate the putative suppressor molecules, an aliquot of absorbed serum, from DNP-BP-treated, DNP-OA-immunized mice was passed through a Sephadex G-100 column. Fractions were eluted and pooled as illustrated in Fig. 10. The fractions were concentrated to the initial volume applied to the column (3.0 ml), were dialyzed against PBS (0.015 M, pH 7.4) and

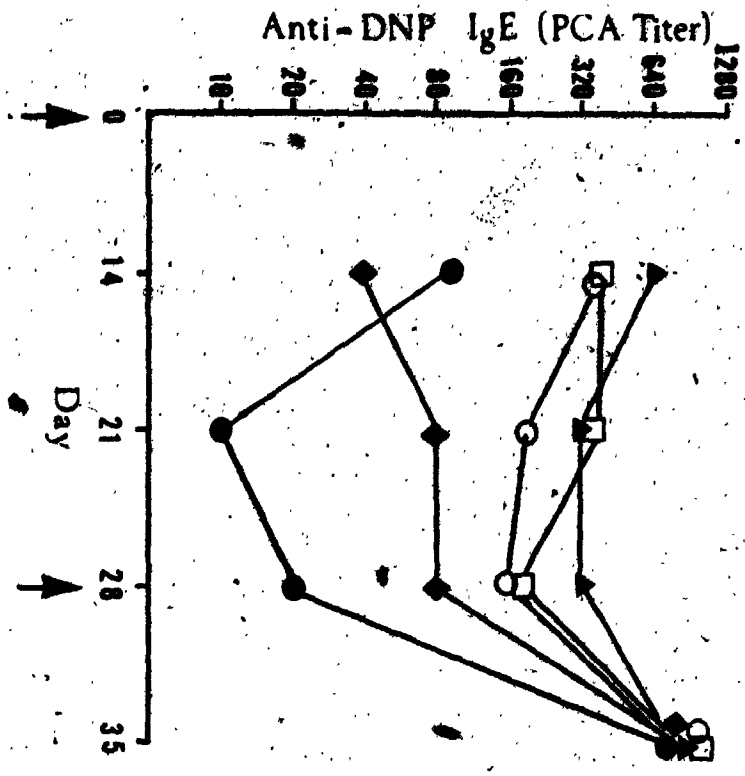
Figure 10. Sephadex G-100 elution profile of serum from DNP-BP-treated mice. Three milliliters of DNP-sepharose and OA-sepharose absorbed serum from DNP-BP treated and DNP-OA alum immunized mice were applied to a 2.5 x 86 cm sephadex G-100 column. Fractions were eluted with PBS and pooled as indicated (horizontal bars). The column was calibrated with 10 mg each of bovine serum albumin (BSA, 69,000d), ovalbumin (OA, 45,000d) and cytochrom c (cyt.c, 12,500d). The arrows indicate the position of each marker.

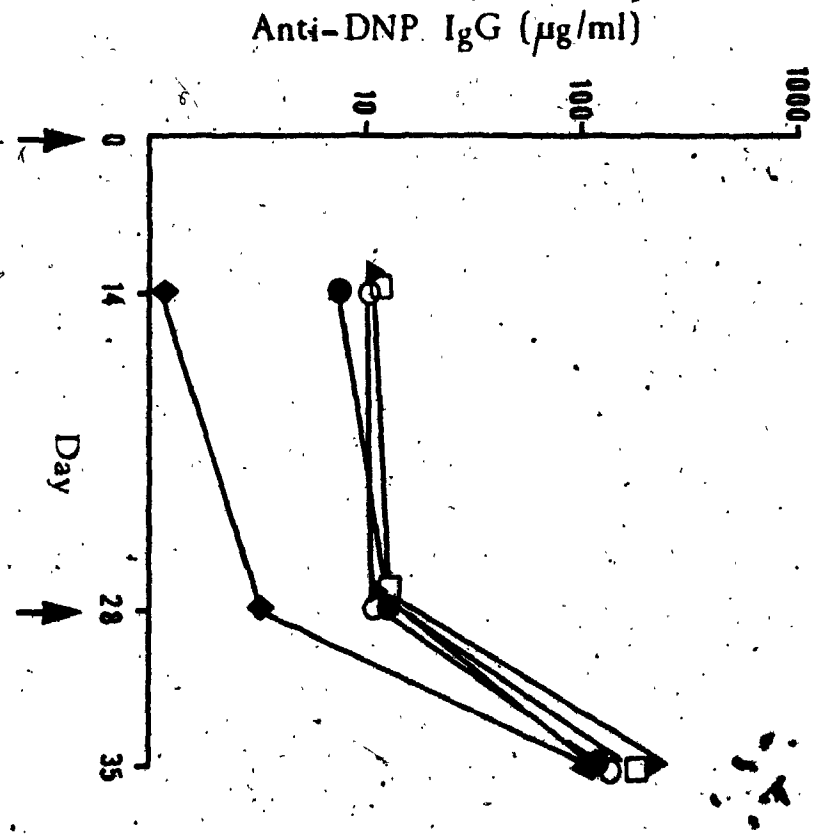

Vo
2.00
1.00
OD 280nm
BSA
OA
Cyt.C
Fraction 1
Fraction 2
Fraction 3
Fraction 4
30
40
50
60
70
80
90
100
110
TUBE NUMBER

injected into mice. Each fraction was injected in 0.25 ml volumes, i.v. twice at 24 hr. interval. Priming with 2 µg DNP-OA in alum was done 12 hrs. after the first injection of fractions, and booster was performed 28 days later. Anti-DNP IgE and IgG antibodies were determined on day 14, 21, 28 and 35. The suppressive effect on anti-DNP IgE response was clearly evident in fraction 1 (Fr. 1, void volume), and fraction 4 (Fr. 4, mol. wt. 12.5×10^3 d), as seen in Fig. 11. Sera of mice which were injected with Fr. 1 or Fr. 4 had a PCA titer of 1:80 and 1:40 respectively on day 14, while mice injected with PBS showed a titer of 1:320 on the same day. The IgE PCA titer in Fr. 1-treated group dropped to 1:10 on day 21, while in Fr. 4-treated mice it was 1:80. Overall the anti-DNP IgE response of Fr. 1-injected recipients was more suppressed than Fr. 4. Fractions 2 and 3 failed to suppress the IgE response. After booster (day 35) none of the groups was suppressed (PCA titers 1:1280). When anti-DNP IgG antibodies were determined it was noted that on days 14 and 28 the group receiving Fr. 4 had slightly lower antibody levels than all the other groups. The differences were, however, small and probably not significant.

These results showed that the absorbed serum of DNP-BP-treated mice contains 2 components with suppressive activity, which differ in molecular weight. However, under these experimental conditions the suppressive effect of both fractions (Fr. 1 and Fr. 4) was reversible and like the effect of the unfractionated AS allowed a normal response following secondary immunization.

Figure 11. Effect of serum fractions obtained by sephadex gel filtration on the anti-DNP response of mice. Each fraction was injected i.v. in separate groups of mice, twice, 0.25 ml each time, at 24 hr. interval. Mice were immunized 12 hr. after the first i.v. injection and boosted 28 days later with 2 μg DNP-OA alum. Anti-DNP IgE and IgG antibodies were detected on the days indicated. Fraction 1 (●), Fraction 2(○), Fraction 3 (▲), Fraction 4 (◆), PBS (□). Arrows indicate days of immunization with DNP-OA alum.


Anti-DNP IgE (PCA Titer)
10
20
40
80
160
320
640
1280
0
14
21
28
35
Day

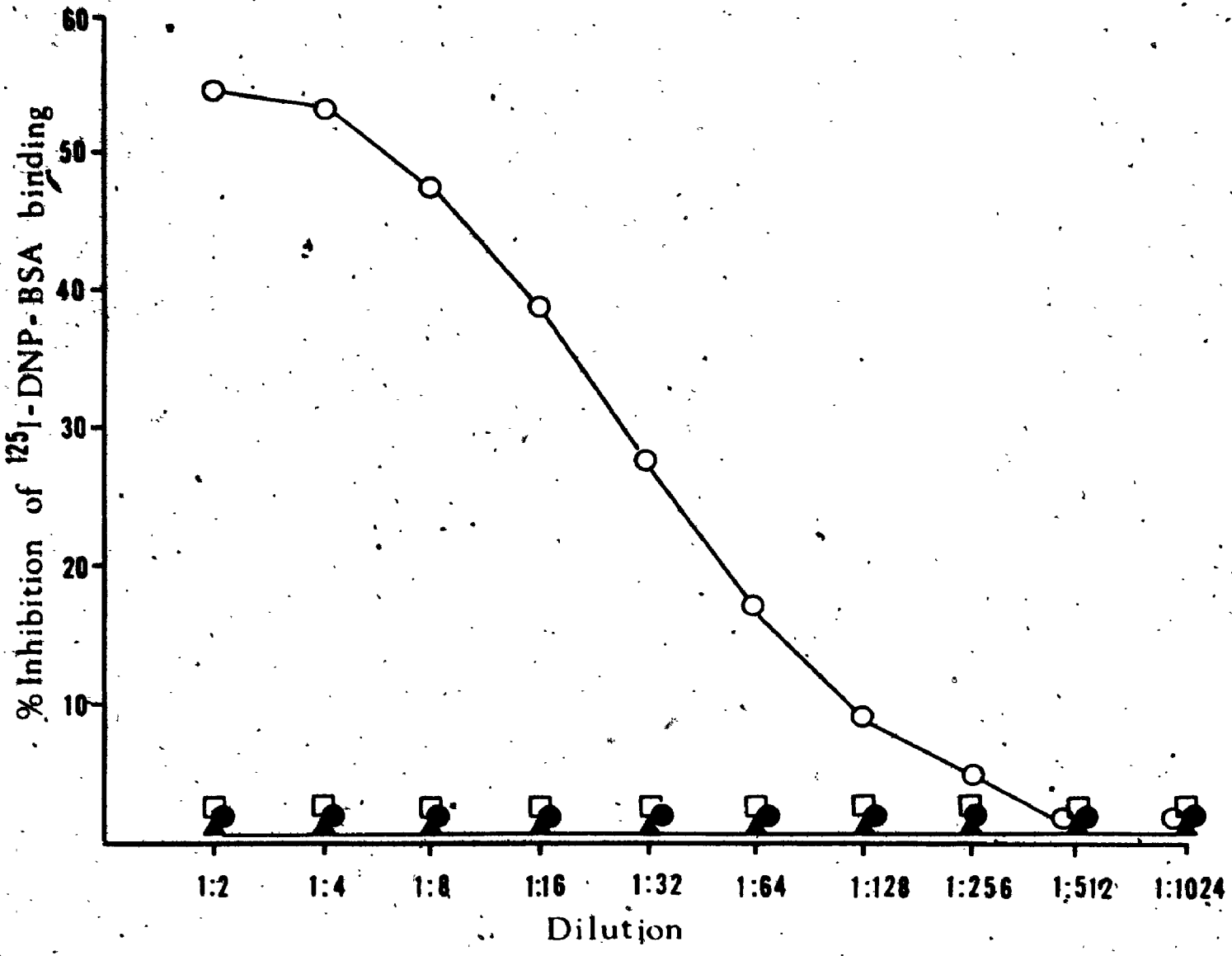

Anti-DNP IgG (μg/ml)
10
100
1000
0
14
28
35
Day

4.11 Demonstration of Auto-Antiidiotypic Activity in the Sera of DNP-BP-Treated Mice:

From the results presented in Fig. 10 and Fig. 11 it became obvious that the suppressive component present in Fr. 1, eluted together with the peak containing the bulk of the immunoglobulins. Since anti-DNP and anti-OA antibodies have been removed prior to gel filtration, the suppression observed could be attributed to the presence of auto-antiidiotypic (aId) antibodies directed against an anti-DNP idiotype.

In order to test this assumption a solid phase RIA-inhibition was carried out as described in 'Materials and Methods' (section 3.22). The source of idiotype (Id) was the same batch of anti-DNP antibody from day 21 sera of mice treated with DNP-BP and immunized with DNP-OA in alum. Idiotype-coated wells of a microtiter plate were allowed to react with twofold serially diluted absorbed serum (AS) obtained from DNP-BP-treated mice, with nonabsorbed serum (NAS) from the same animals, with Fr. 4 or with normal mouse serum (NMS). Following the appropriate incubations and washings, wells were incubated with a constant amount (20 ng/well) of ^{125}I-DNP-BSA. The radioactivity bound/well was counted and the extent of inhibition of ^{125}I-DNP-BSA binding to the anti-DNP antibodies (Id) was plotted against the dilution of serum used as a source of anti-idiotypic activity. As seen in Fig. 12, the AS demonstrated a dose dependent inhibition of ^{125}I-DNP-BSA binding to anti-DNP antibodies, providing clear evidence that the suppressive activity present in the AS is capable of specifically reacting with anti-DNP antibodies (Id). A dilution of 1:2 gave 54.5% inhibition with the highest effective

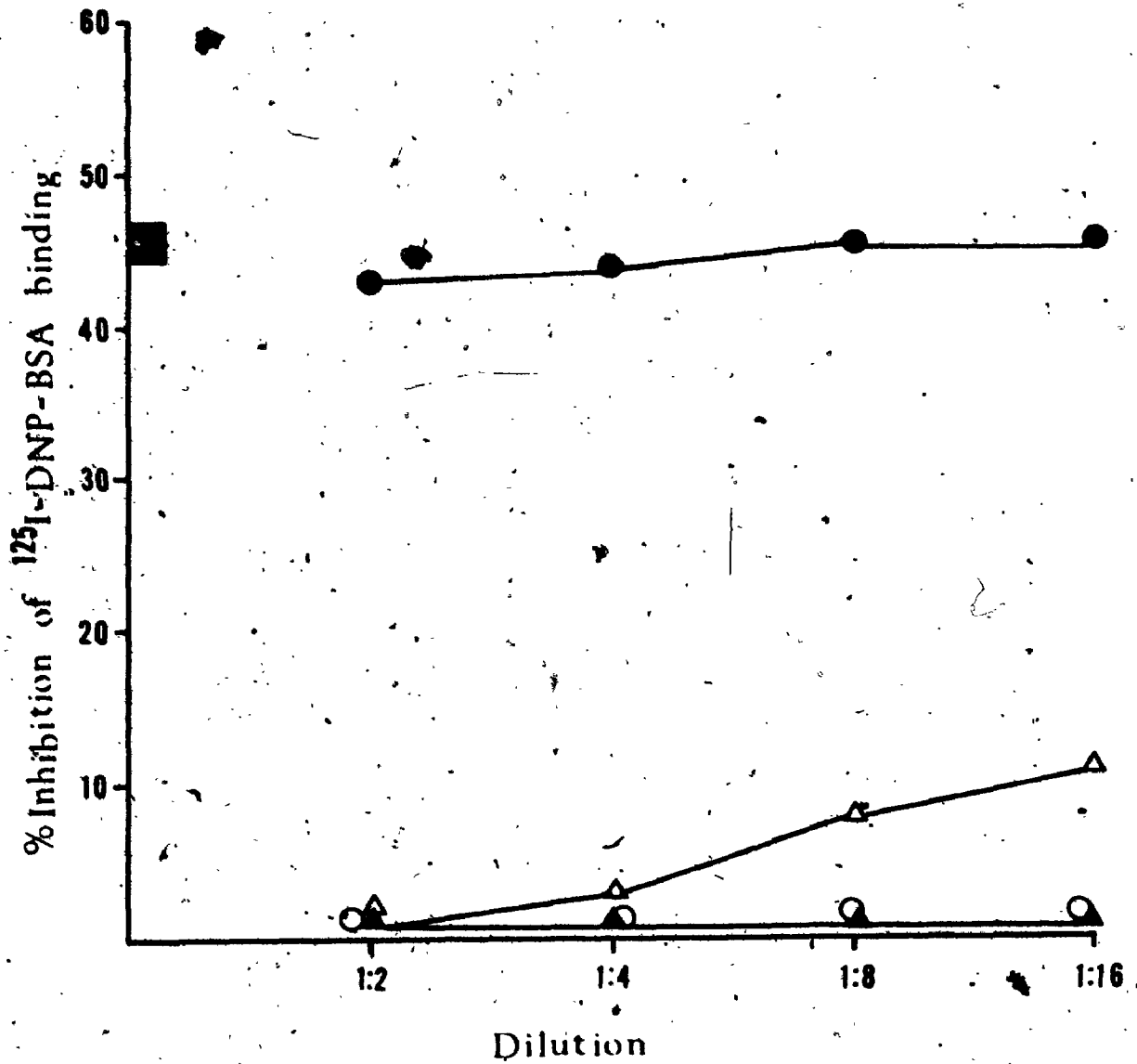
Figure 12. Demonstration of auto-anti-idiotypic activity in the serum of DNP-BP-treated mice. Wells of a microtiter plate were coated with anti-DNP antibodies (Id) and reacted with two fold serial dilutions of absorbed serum (○), nonabsorbed serum (●) or sephadex G-100 Fr. 4 (▲) from DNP-BP-treated, DNP-OA immunized mice, or with equivalent dilutions of normal mouse serum (□). Following incubation and washings, the wells were allowed to react with a constant amount (20 ng/well) of ^{125}I-DNP-BSA. Results are expressed as % inhibition of ^{125}I-DNP-BSA binding to anti-DNP antibodies (Id).


% Inhibition of ^{125}I-DNP-BSA binding
60
50
40
30
20
10
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
1:1024
Dilution

dilution of 1:256. It is important to note that Fr. 4 which also demonstrated some suppressive activity (Fig. 11) failed to inhibit the binding of ^{125}I-DNP-BSA in this assay. The nonabsorbed serum, was also without inhibitory activity. It must be remembered, however, that the nonabsorbed serum contained relatively large amounts of anti-DNP antibodies (Id) and, therefore, the putative aId antibodies would have been bound to the idiotype, and probably would have been unavailable to inhibit the binding of ^{125}I-DNP-BSA to the anti-DNP antibody-coated wells. Based on this assumption we also predicted that excess anti-DNP antibody would be present in the serum in free form (unbound to anti-Id antibodies) and, therefore, in a position to bind additional aId. This prediction was tested by allowing nonabsorbed serum to interfere with the inhibition of ^{125}I-DNP-BSA binding to idiotype by anti-idiotypic antibodies (absorbed serum).

Thus serial two fold dilutions of NAS were mixed with a constant amount of aId (AS) and the mixture was added to Id (anti-DNP antibodies)-coated microtiter wells. Controls consisted of AS mixed with serial dilutions of normal mouse serum (NMS), of NAS alone, of NMS alone, and of AS (aId) alone. After incubation and washings, ^{125}I-DNP-BSA was added to all wells and the amount of radioactivity bound to the anti-DNP antibodies (Id) was determined as described above. The results presented in Fig. 13 show that mixing of absorbed serum with nonabsorbed serum resulted in complete abolition of the inhibitory capacity of the AS. This effect was dose-dependent as the inhibitory activity of the AS started to reemerge as the dilution of the NAS increased. On the other hand, premixing of AS with normal

Figure 13. Blocking of anti-idiotypic activity by nonabsorbed serum from DNP-BP-treated mice. Microtiter wells were coated with affinity-purified anti-DNP antibodies (Id). Hundred microliters volumes of absorbed serum (AS) from DNP-BP-treated mice, diluted 1:2, were added to 100 µl of two fold serial dilutions of nonabsorbed serum (NAS) from DNP-BP-treated mice (△), or to normal mouse serum (●). The mixture was then added to the idiotype-coated wells. Hundred microliters volumes of serial dilutions of normal mouse serum alone (⊙), of nonabsorbed serum alone (▲) or 100 µl of a 1:4 dilution of anti-Id (absorbed serum ■) served as negative and positive controls respectively, and were added separately to idiotype-coated wells. Following incubation and washings wells were allowed to react with ^{125}I-DNP-BSA (20 ng/well), and the inhibition of ^{125}I-DNP-BSA binding to anti-DNP antibodies was calculated by counting the bound radioactivity in the wells.


60
50
40
30
20
10
% Inhibition of ^{125}I-DNP-BSA binding
1:2
1:4
1:8
1:16
Dilution

mouse serum did not lead to a reduction in inhibitory capacity. Neither NAS nor normal mouse serum alone inhibited the activity of ^{125}I-DNP-BSA to anti-DNP antibodies at any dilution tested.

These results bring circumstantial evidence in support of the notion that the anti-idiotypic antibodies present in the nonabsorbed serum of DNP-BP treated mice circulate in the form of loose complexes with anti-DNP antibodies and that the anti-DNP antibodies are in sufficient excess to block the inhibitory activity of additional, anti-Id antibodies. As the dilution of the NAS increases the free idiotype concentration decreases accordingly, allowing the recovery of the inhibitory activity of the added anti-iodiotype.

5. DISCUSSION

IgE antibody responses have been shown to be exquisitely sensitive to both positive and negative T cell regulatory effects, and to antibody feedback mechanisms (Katz et al., 1974; Tada, 1975; Ishizaka, 1976; Ishizaka and Ishizaka, 1978; Ovary et al., 1978; Katz, 1978b; Kishimoto, 1982). In contrast to antibody responses of other immunoglobulin classes, the IgE antibody response is highly dependent on adjuvant. In the past, efforts have been made to down-regulate the IgE antibody response by immunological adjuvants, known to potentiate IgG antibody responses. The use of hapten-conjugated Mycobacterium (DNP-MYC and PC-MYC) or hapten-conjugated muramyldipeptide (MDP) in mice has been well documented, (Kishimoto et al., 1976, 1979, 1981; Sugimura et al., 1982).

Since adjuvants commonly used to potentiate IgE antibody responses in laboratory animals (alum and BP vaccine) have not been used before to modulate the immune response, we decided to study the immune response of hapten-conjugated Bordetella pertussis organisms in mice.

We showed that CBA/J mice immunized with hapten-adjuvant conjugate (DNP-BP) failed to mount a primary or a secondary anti-DNP IgE response regardless of whether an additional adjuvant 'alum' was used. Occassionally low levels of IgE antibodies were detected. On the other hand, mice developed excellent anti-DNP IgG responses.

It has been shown by others that the production of IgE antibodies is highly dependent on certain factors such as the dose of antigen used for immunization, adjuvant employed, and the strain of mice used for immunization (Revoltella and Ovary, 1969; Levine and

Vaz, 1970; Vaz et al., 1971). It has also been reported that immunogenicity of a hapten-carrier conjugate also depends on its hapten/carrier ratio (Quijada et al., 1974). In our experimental system, injecting various amounts or by changing the hapten/carrier ratio of DNP-BP conjugates there was no significant change in the pattern of low-responsiveness for IgE in CBA mice.

It is well established that the development of IgE antibody responses differs in magnitude among various inbred strains such that some strains exhibit vigorus IgE responses, whereas other strains produce very low or no IgE antibodies (Revoltella and Ovary, 1969; Levine and Vaz, 1970; Chiorazzi et al., 1976, 1977; Watanabe et al., 1976). Immunization of various strains of mice with DNP-BP revealed that this lack of immunogenicity in CBA mice for the induction of IgE response was not an intrinsic attribute of the DNP-BP conjugate, since it induced high levels of IgG antibodies in all strains studied, as well as IgE antibodies in strains carrying the $H\text{-}2^b$ and $H\text{-}2^d$ haptotype (Fig. 3). The strains carrying the $H\text{-}2^a$ and $H\text{-}2^k$ haptotype but sharing the same genes at K region and IA, IB, IJ and IE subregions were found to be low IgE responses for DNP-BP. The low IgE levels detected in CBA mice, however, never exceeded a PCA titer of 1:80. This is consistent with observations by others indicating that low responder mice may show PCA titers of 1:80 but not higher (Katz et al., 1979b). DNP-BP then, as any other conventional antigen, follows an immunogenicity pattern clearly distributed among low and high responder phenotypes.

As demonstrated repeatedly by others in the past, the low responder phenotype is often associated with the concomitant presence of an active suppressor mechanism that operates in an immunoglobulin class-selective (Watanabe et al., 1976; Chiorazzi et al., 1976, 1977) or non-selective fashion (Kapp et al., 1974). It has also been shown previously that treatment of low responder mice with low dose x-irradiation or moderate dose of cyclophosphamide prior to immunization results in the depletion of the suppressive mechanism and thus mice may change their status from low to high responder phenotype (Katz, 1978b). However, in our experimental system pretreatment of mice with such physical and chemical agents failed to enhance their ability to mount a primary IgE antibody response following DNP-BP immunization but after booster weak secondary responses were determined which were transient and within a week declined to very low levels. Similar findings were reported by Dessein et al., (1979), where treatment of nonresponder mice with cyclophosphamide or low dose irradiation failed to affect the anti-GAT IgE response.

Besides its ability to induce excellent IgG responses, administration of DNP-BP to CBA mice did not result in IgE antibody production even when an additional adjuvant (alum) was injected along with the antigen. It is interesting to note, however, that DNP-specific IgE B-memory cells were generated, even when DNP-BP was administered in saline. This was clearly demonstrated when such B cells were removed from their original environment and cotransferred with appropriately primed T-helper cells into unprimed, x-irradiated mice. Under these circumstances the anti-DNP IgE response appeared as

a true secondary response. Furthermore when spleen cells from DNP-BP-treated donors were transferred into normal recipients or when DNP-BP-treated mice were boosted *in situ* with DNP-OA (alum) an accelerated primary anti-DNP response (on day 7 instead of day 14) was obtained. These findings were in complete agreement with those obtained by Hamaoka *et al.*, (1973, 1974) and Katz *et al.*, (1974), who showed that mice immunized with antigen in CFA did not mount an IgE response but developed IgE B-memory cells (as well as T-helper cells) which could collaborate with an appropriately primed T-helper cell and mount an IgE response when removed from their host environment and challenged with the relevent antigen. In our studies DNP-specific IgE B-memory cells were produced also when conventional hapten-carrier conjugates such as DNP-ASC-1 or TNP-KLH were injected under similar conditions. Peculiar to DNP-BP treatment, however, was the sharp decrease in IgE levels, occuring from two weeks after booster with DNP-OA. The decrease in IgE responses observed in unsubstituted BP-treated, DNP-OA challenged mice (Fig. 4) was never significant with respect to untreated control. This difference between DNP-substituted and unsubstituted BP could be explained by assuming: a) that both DNP-BP and BP could initiate a chain of events leading to the establishment of a suppressor circuit and to the release of IgE-selective suppressor molecules, b) that in order to complete the circuit i.e. to permit the effective release of these molecules, a second exposure to antigen is required and c) that the second antigen must share, with the first, common antigenic determinants. Similar requirements for shared determinants have been demonstrated by

Kishimoto et al., (1976, 1978) and Suemura et al., (1977) in IgE-selective, antigen-nonspecific suppression initiated by preadministration of DNP-Mycobacterium in mice.

An intriguing finding was the early short-lived but intense IgE response taking place both in situ and in adoptively transferred recipients after DNP-OA booster and preceeding demonstrable suppression (Fig. 4, Table V and Table VI). The delayed suppression suggested that the induction of the suppressive mechanism could be a multi-step process and that the kinetics of production of the final effector cell or molecule is slower than the synthesis and release of the IgE antibodies themselves. An alternate explanation is that the effector arc of the suppressor circuit would not be triggered unless a defined concentration of hapten-specific IgE and/or IgG antibodies was present. In fact in a series of elegant in vitro manipulations with rat lymphoid cells, Yodoi and Ishizaka (1979, 1980) and Hirashima et al., (1980, 1981a,b,c, 1982) have demonstrated that both potentiation and suppression of IgE depended on the production of IgE-binding factors and that under certain circumstances rat IgE had to be present in culture for the production of the IgE-binding factors. However, we felt that the requirement for IgE could not be the only condition necessary for the suppression, because immunization of CBA mice with DNP-BP usually failed to induce detectable serum levels of IgE antibodies but still upon booster with DNP-BP the anti-DNP IgE response remained suppressed. The possibility remains, however, that low, undetectable levels of IgE, sufficient to trigger the production of IgE-binding factor (with suppressor activity) were produced.

It is well established that production of antibodies is under the strict control of different subpopulations of T cells. Suppressor T cells play a very important role in the regulation of antibody production (Gershon and Kondo, 1971; Gershon, 1974; Basten et al., 1977). The down-regulation of IgE antibodies by suppressor T cells has been reported in many laboratories (Okumura and Tada, 1971; Takatsu and Ishizaka, 1976; Watanabe et al., 1976; Chiorazzi et al, 1976, 1977; Kishimoto et al., 1976; Ovary et al., 1978). As a common practice the mediation of suppression via suppressor T cells has been shown by trasferring the spleen cells of donor mice into syngeneic recipients (Okumura and Tada, 1971; Kishimoto et al., 1976; Takatsu and Ishizaka, 1976; Ovary et al., 1978; Schwenk et al., 1979).

However, our efforts to transfer the classical pattern of suppression by spleen cells from DNP-BP-treated donors were only marginally successful. A meager and delayed, but statistically significant suppression was detected on 21 days following cell transfer. This slow and weak suppressive effect mediated by adoptively transferred spleen cells from DNP-BP-treated donors, coincides with the results obtained by Watanabe and Ovary, (1976) in a completely different experimental system. They successfully manipulated the low IgE responder SJL mice to mount persistant IgE responses and showed the termination of IgE production by injecting spleen cells from normal SJL mice. The suppression observed following adoptive transfer was quite slow since it appeared 3 weeks after the cell transfer. This seems to be a situation unlike other systems where the suppression is always immediate (Okumura and Tada, 1971; Kishimoto et al., 1976; Takatsu and Ishizaka, 1976). However,

the delayed suppression reported by Watanabe et al., (1976) was stronger than detected in our experimental system. When DNP-BP-treated mice were used as recipients of OA or DNP-OA primed spleen cells and challenged with DNP-OA alum a burst of IgE antibodies on day 7 after cell transfer was followed by a strong suppression of IgE response. This suggested that the environment present in DNP-BP-treated recipients does not affect immediately the capability of IgE forming cells to mount an IgE response because the day 7 anti-DNP IgE response in DNP-BP-treated recipients was equivalent to that observed in untreated control groups. On the other hand, TNP-KLH-treated recipients mounted a higher IgE antibody response on day 7 as compared to the DNP-BP-treated or untreated group, showing a positive contribution of the recipient in IgE production. It appears that some unknown factor or molecule is present in DNP-BP-treated mice either in insufficient amounts or at an ineffective stage and requires more time to become effective (suppressive). Treatment of the recipients with other hapten-carrier conjugates (TNP-KLH) also resulted in decreased PCA titers from day 7 to day 14 (four fold decrease) following transfer of OA primed spleen cells but despite this decrease the day 14 titers were comparable to the control group, where recipients were not treated. Experiments performed in DNP-BP-treated mice, which were then challenged with DNP-OA, provided evidence for a suppressive environment, with respect to the persistance of the IgE antibody response. However, the induction of the IgE response was not impaired. It appears that the suppressive mechanism operating in DNP-BP-treated mice following challenge with DNP-OA (hapten on a heterologous carrier) is different from the one

operating when DNP-BP primed mice are boosted by the same conjugate (DNP-BP). These two different suppressive pathways differ in their kinetics. In the case of DNP-BP/DNP-BP system, suppression acts upon an early stage. Whereas in the case of DNP-BP/DNP-OA, the suppression is active in later stages of the IgE response. This was observed both in situ and in adoptive transfer studies. In both cases the suppression was delayed and could be detected 21 days following DNP-OA challenge. However, the extent of suppression was always stronger in situ, than in experiments where spleen cells from DNP-BP-treated donors were transferred into normal recipients. The exact reason why priming and booster with DNP-BP conjugate is so effective in exerting a suppressive effect on IgE production in the presence of strong IgG antibody production is not clear. It is possible that IgE unresponsiveness to DNP-BP is mainly carrier dependent although a humoral (serum) suppressive molecule such as anti-carrier (BP) IgG antibody could also be involved. In the case of priming with DNP-BP and booster with DNP-OA the requirement for homologous carrier (BP) is not satisfied and carrier-reactive suppressor cells or anti-carrier IgG antibodies are not recalled. The delayed suppression observed in DNP-BP/DNP-OA system is hapten-specific. Challenge of DNP-BP-treated mice with OA (alum) failed to suppress anti-OA IgE antibody responses. Furthermore, immunization of DNP-BP-treated, DNP-OA challenged, mice with an irrelevent antigen, KLH (alum), at the time of evident anti-DNP IgE suppression (day 21) did not suppress anti-KLH IgE production. These findings preclude the possibility that the IgE suppression mechanism operating in DNP-BP-treated, DNP-OA challenged mice has any similatiry to the DNP-Myc,

DNP-OA system described by Kishimoto et al., (1978). In their system the suppressive mechanism initiated by DNP-Myc administration, could be restimulated by DNP on a heterologous carrier but the suppressive effect was antigen non-specific.

IgE-selective suppressive mechanisms described by several investigators, appear to show many common characteristics. Mostly they are antigen-nonspecific, naturally occuring in low IgE responder mice (Watanabe et al., 1976; Chiorazzi et al., 1976, 1977), can be induced in low and high responder mice and in rats by the administration of adjuvant containing Mycobacterium and water-in-oil emulsion (Kishimoto et al., 1978; Katz and Tung, 1978; Hirashima et al., 1982). The suppression is transferable by T cells and is abrogated by low doses of x-irradiation or by pretreatment with cyclophosphamide (Watanabe et al., 1976; Chiorazzi et al., 1977). The serum, cell-free supernatants and T-T cell hybrids derived from lymphoid cells of CFA-treated or hapten-Mycobacterium-treated animals contain (release) soluble molecules that suppress very effectively the IgE response either in adoptive transfer or in tissue culture responses (Suemura et al., 1977; Kishimoto et al., 1978; Watanabe et al, 1978; Tung et al., 1978; Hirashima et al., 1981a; Sigimura et al., 1982). In may instances both IgE suppressor and IgE enhancing molecules coexist and their individual presence can be demonstrated only after affinity chromatography on lectin-conjugated insoluble matrices (Katz et al., 1979; Hirashima et al., 1980).

The failure of the IgE antibody response to occur in CBA mice after immunization with DNP-BP did not fit well into the recognizable pattern described for either CFA or for DNP-MYC. It is induced by an

adjuvant which unlike CFA or IFA is known to potentiate the IgE responses (Mota, 1964; Hirashima et al.; 1981a; Iwata et al., 1983), it is delayed, follows after a transient burst of IgE antibody and transfer to syngeneic, unprimed mice, is only marginally effective and always delayed. T cells are apparently not directly involved since, transfer of T-enriched spleen cells from DNP-BP-treated donors to normal recipients failed to show any suppressive effect on the IgE production. Furthermore depletion of such T cells by anti-Thy 1.2 and complement treatment could not remove the suppression observed on day 21 upon transfer of both populations of spleen cells (T and B cells together).

It was interesting to note that the appearance of IgE selective suppression was always associated with high anti-DNP IgG levels and thus suggested the possibility of an IgG-antibody mediated feedback regulatory mechanism in our experimental system. It is generally believed that like the production of other biologically active molecules, production of antibody is also a self-limiting process (Rawley and Fitch 1964; Uhr and Moller 1968; Graf and Uhr, 1969; Sinclar, 1969). The regulation of IgE antibody response in an antigen-specific manner by passively administered IgG antibodies has been demonstrated in rats by Tada and Okumura (1971). Ishizaka and Okudaira (1972) reported that passive transfer of anti-OA IgG antibodies in mice suppressed the induction of both IgE and IgG antibodies against OA. However, the administration of antibodies did not terminate pre-existing IgE antibody formation nor did they suppress secondary antibody responses. In contrast Tada and Okumura

(1971) reported that in rats passively transferred antibodies preferentially suppressed not only the induction of IgE antibody response but also terminated an ongoing IgE response.

In our system passive transfer of the serum obtained from DNP-BP-treated mice (injected with 10^9 DNP-BP 16, 14 and 12 days prior to bleeding), failed to suppress the induction or to terminate the pre-existing antibody response. However, the same volume of serum obtained from DNP-BP-treated, DNP-OA challenged mice (21 days after DNP-OA injection) showed very strong suppressive effect on the induction of primary anti-DNP IgE response. The IgG response however, was not much affected. Ishizaka and Okudaira (1972) have shown that after the disappearance of passively transferred antibodies an essentially normal immune response could develop in mice and both IgE and IgG antibodies were detected in the serum 40 days after primary immunization. However, in our system mice passively injected with serum were unable to mount IgE antibody response for at least 6 weeks.

These data together with the results obtained in a comparative study on different strains of mice (where high IgE responders, C57 BL/6, DBA/2 and BALB/C, also mounted extremely high levels of IgG antibodies) suggested that antibody feedback can not be the exclusive mechanism responsible for IgE selective suppression observed in the present experiments in CBA mice.

The possibility that the high IgG levels in the serum of DNP-BP-treated, DNP-OA injected mice were responsible for the passively induced IgE suppression was ruled out when absorbed serum (depleted of anti-DNP and anti-OA antibodies) showed the suppressive effect not

only on the induction but also on an ongoing anti-DNP IgE antibody response (Fig. 9). On the other hand absorbed serum from DNP-CE-treated, DNP-OA injected mice failed to show any suppressive effect under identical experimental conditions and such unabsorbed serum showed marked suppression.

Taken collectively these results strongly argue against the possibility of antibody feedback suppression operating in our experimental system. Two possible mechanisms could then be involved: a) an IgE-selective, antigen-nonreactive suppressor molecule or, b) an auto-anti-idiotypic regulatory mechanism.

The experiment in which absorbed serum (AS) was fractionated on sephadex G-100 column showed that suppressive activity was associated with two serum fractions. Fraction 1, which contained the void volume and Fraction 4, approximating the molecular weight of cytochrome C (12,500 d). Passive transfer of these fractions in normal syngeneic mice affected the induction and persistence of anti-DNP IgE antibodies after primary immunization with DNP-OA. The low molecular weight fraction (Fr. 4) showed some suppressive effect on the induction of IgG antibody response as well but this difference as compared to other groups was not very significant.

The presence of the suppressive activity in an immunoglobulin-containing fraction (Fr. 1) provided sufficient circumstantial evidence to suggest that auto-anti-idiotypic antibodies were involved. More convincing evidence for the association of anti-idiotypic activity with Fr. 1 was provided by the inhibition of RIA where the binding of ^{125}I-DNP-BSA to anti-DNP antibodies (idiotype) was prevented by incubating the idiotype-coated wells first with the

serum containing the putative anti-idiotypic (aId) activity. This inhibition was directly dependent on the concentration (dilution) of serum which contained anti-idiotypic activity and was not observed when idiotype-coated wells were incubated with normal mouse serum. It was interesting to note that nonabsorbed serum (NAS, obtained form DNP-BP-treated, DNP-OA challenged mice), failed to react with idiotype (anti-DNP antibodies) and to inhibit the binding of ^{125}I-DNP-BSA to idiotype (Id). However, the same serum after depletion of anti-DNP and anti-OA antibodies (AS) did bind to the anti-DNP antibodies. This can be explained by assuming that the nonabsorbed serum (NAS) contained idiotype in excess of anti-idiotypic antibodies and both coexist, mainly as soluble complexes. Thus when such a serum is allowed to react with idiotype obviously it can not bind to it. Once the serum is absorbed on DNP-BSA-sepharose (and OA-sepharose) immunosorbents the anti-DNP antibodies (idiotype) are removed and anti-idiotypic antibodies are free to bind.

An important question is how to reconcile the presence of idiotype-anti-idiotype complexes in the serum with the observation that after passage of the serum through DNP-BSA-sepharose, anti-DNP antibodies are removed but anti-idiotypic antibodies remain free in the effluent. This can be explained by assuming that the binding affinity of anti-DNP antibodies for DNP-BSA-sepharose is much stronger than the affinity of anti-idiotypic antibodies for the idiotype. Thus when the serum is passed through DNP-BSA-sepharose, the anti-idiotypic antibodies dissociate from the idiotype by the high-affinity-binding hapten. However, the presence of some aId-Id-DNP insoluble complexes on the column can not be ruled out.

The evidence for the assumption that Id and aId coexist in the form of soluble complexes was provided by an experiment in which nonabsorbed serum was serially diluted and mixed with a constant volume of absorbed serum (AS). The constant amount of AS added into each dilution of NAS showed on its own 46% inhibition of ^{125}I-DNP-BSA binding to anti-DNP antibodies (Id) (Fig. 13). However, when the same volume of AS was added to NAS, it was unable to interfere with ^{125}I-DNP-BSA binding to Id. As the NAS was further diluted the inhibitory effect started to reappear. When the same procedure was applied to normal mouse serum the anti-idiotypic activity was present throughout. It was interesting to note that the low molecular weight fraction (Fr. 4) which also demonstrated the suppressive effect in the recipient mice, failed to react with Id, indicating the existance of yet another molecule, which can mediate antigen-specific suppression but is not reactive with antigen.

As far as fraction 1 (Fr. 1) is concerned there is ample circumstantial evidence to indicate that it contains anti-idiotypic antibodies i.e. (a) the gel filtration profile (the suppressive activity was present in the void volume where all immunoglobulins were present), (b) the removal of anti-DNP and anti-OA antibodies from the serum with suppressive activity fails to eliminate the suppressive property of the serum, arguing strongly against the IgG antibody feedback as an exclusive mechanism of suppression. (c) the suppressive serum (AS) specifically reacts with anti-DNP antibodies and inhibits the binding of iodinated DNP-BSA to anti-DNP antibodies in a concentration-dependent manner. (d) this suppressive serum suppresses anti-DNP IgE responses <u>in vivo</u> upon passive transfer into

normal or primed mice. It may, therefore, be reasonable to suggest that the antigen-specific and IgE-selective suppression observed in DNP-BP-treated, DNP-OA challenged CBA/J mice works via autologous anti-idiotypic antibodies.

The ability of an animal to mount an immune response against autologous (Rodkey, 1974; Kluskens and Kohler 1974; Bankert and Pressmann, 1976; Goidl et al., 1980a, 1980b, 1983) and isologous idiotypes (Sirisinha and Eisen, 1971; Eichmann, 1972; Sakato and Eisen, 1975; Blaser et al., 1979, 1980) has been well documented. Furthermore, idiotypic-anti-idiotypic interactions have been shown to play an important role in the regulation of the immune responses in laboratory animals (Hart et al., 1972; Binz and Wigzell, 1976; Yamamoto et al., 1979; Brown and Rodky, 1979; Reth et al., 1981; Blaser and de Weck, 1982). Thusfar, three different approaches have been employed by various workers to induce anti-idiotypic antibodies.

The first approach involved hyperimmunization of animals with a given (usually T-independent) antigen. Kluskens and Kohler (1974) and Cosenza (1976) have previously reported that hyperimmunization of mice with pueumococcal antigen containing phosphorylcholine induced the production of anti-idiotypic antibodies or idiotype-specific plaque forming cells (PFC). Similarly, such anti-idiotypic antibodies have also been produced in various other antigenic systems (Schrater et al., 1979; Fernandez and Moller, 1979; Brown and Rodkey, 1979; Sy et al., 1979; Dzierzak et al., 1980, 1981).

The second approach involved immunization of animals with isologous or heterologous idiotypes in combination with adjuvant, which usually resulted in high levels of anti-idiotypic antibodies

(Granto et al., 1974; Sakato and Eisen, 1975; Bona and Paul, 1979; Blaser et al., 1981b).

The third approach made use of the hapten reactive T helper cells for the induction of anti-idiotypic antibodies following immunization with hapten-conjugated idiotypes (Iverson, 1970; Yamamoto et al., 1980).

The results obtained during our study indicate that anti-idiotypic antibodies may appear during the course of normal immunization using a T-dependent antigen and that they are potentially capable of markedly suppressing the anti-DNP-IgE response. It has been shown by others that the expression of most idiotypes among immunoglobulins is genetically linked to H-chain allotypes which are controlled by the immunoglobulin constant heavy locus and antibodies of different classes bear similar or identical idiotypes (Imanishi et al., 1975; Weigert and Potter, 1977; Dessein et al., 1980; Hirano et al., 1983). However, the studies done in mice to elucidate the regulatory effect of anti-idiotypic antibodies specific for phosphorylcholine (PC) or Benzylpenicilloyl (BPO) haptens have suggested that IgE antibody formation is particularly susceptible to suppression by the anti-idiotypic antibodies (Blaser and de Weck, 1982). The high degree of susceptibility of the IgE system could be attributed to its low concentration in the serum, to its exclusively T-dependent regulation, to its short half-life or, to a special susceptibility of cells regulating and/or producing IgE

antibodies to the activity of the anti-Id antibodies. Thus, IgE selective suppression in our system is consistent with findings of Blaser and de Weck (1980, 1981a,b).

Anti-DNP antibody response is generally considered as idiotypically heterogeneous (Askonas et al., 1970). Schrater et al., (1979) reported a decline in BALB/C and AKR mice of the anti-TNP plaque forming cell (PFC) response between 4 and 7 days after immunization with TNP-Ficoll. They attributed this decline to putative anti-idiotypic auto-antibodies which were bound on the surface of anti-TNP antibody-secreting cells, but they were unable to provide information about the idiotype(s) against which the putative anti-Id antibodies were directed (Goidl et al., 1979). However, Dzierzak et al., (1980, 1981) reported that following booster with DNP-OA, BALB/C mice produced anti-DNP antibodies and PFC belonging to the M460 idiotype. Idiotype 460 is represented in all immunoglobulin classes including IgE isotype for a transient period of time and is considered a dominant idiotype. Furthermore, they concluded that the regulation of isotype expression is independent of the regulation of idiotype expression in this system.

In the DNP-BP/DNP-OA system at the present time, we are unable to provide any information regarding the number of idiotypes and their prevalence in the serum of CBA mice, but we believe, however, that the IgE antibodies may share common idiotype(s) with other Ig classes. In this way the capacity of DNP-BP to stimulate high levels of DNP-BP IgG antibodies may be primarily responsible for the anti-idiotype-mediated IgE selective suppression observed.

We have shown in our experiments that passive administration of absorbed serum (containing putative anti-Id antibody) in unprimed and DNP-OA primed syngeneic mice results in the IgE-selective, DNP-specific suppression. Although this suppression is only partial, the IgE levels in the group receiving AS obtained from DNP-BP-treated-DNP-OA challenged mice are significantly lower than detected in recipients of NMS or in noninjected control. Concomitant administration of anti-Id antibodies and of antigen leads to a temporary depression of the primary response in the BPO system but does not cause the long-lasting suppression observed in animals actively producing anti-Id antibodies (Balser et al., 1980). Furthermore, these authors have shown that a single i.v. injection of anti-BPO anti-idiotypic antibodies in BALB/C mice specifically suppressed an already established IgE response for 2-3 weeks (Blaser et al., 1980), while an established anti-PC IgE response is suppressed in BALB/C mice by two i.v. injections of anti-T15 anti-idiotypic antibodies. The suppression induced in this way was maintained, despite repeated immunizations with antigen in alum (Blaser et al., 1979). Similar results by passive administration of antisera containing anti-idiotypic antibodies were reported in guinea pigs with various antigens. Passive administration of serum in guinea pigs obtained from animals immunized with syngeneic purified anti-BPO-BGG antibodies resulted in a marked suppression of IgE antibody response for 3-4 weeks (Geczy et al., 1978). The suppression of the IgE antibody response by actively producing anti-idiotypic antibodies has been shown in various experimental systems. BALB/C mice, forced to make anti-T15 idiotype antibodies by multiple injections of T15

idiotype and then immunized with PC-KLH in alum showed, highly suppressed anti-PC IgE responses. Similarly, BALB/C mice immunized with syngeneic anti-BPO IgG antibodies were unable to produce anti BPO IgE upon repeated injections of BPO-OA (Blaser and de Weck, 1982).

However, in the DNP-BP/DNP-OA system the suppression of the anti-DNP IgE response occured in the process of normal immunization with an extraneous antigen, and not as a result of intentional immunization with idiotype.

Our studies showed that normal spleen cells incubated in vitro for 18 hrs. with the serum containing the putative anti-idiotypic antibodies, in the absence of antigen and transferred into x-irradiated recipients, had suppressed primary but not secondary anti-DNP IgE responses following challenge of the recipient with DNP-OA in alum. On the other hand normal mouse serum had no effect.

By taking advantage of the idiotypic homogeneity in anti-PC antibody in BALB/C mice and the easy availability of TEPC-15 myeloma protein (T15), the effect of anti-idiotypic antibodies was studied in vitro (Kim, 1979). The presence of anti-T15 Id antibody for at least 2 days was necessary to achieve a full level of irreversible suppression of anti-PC production in the presence of relevant antigen R36a (Kim, 1979; Massey and Kim, 1981). Moreover, when spleen cells were treated (in vitro) with anti-idiotypic antibodies in the absence of relevant antigen, they showed reversible tolerance in vitro culture (Kim, 1979). Furthermore, cultures containing either unfractionated spleen cells or B cell-enriched populations that had been pre-exposed to antigen for longer than 24 hrs. in the absence of

anti-idiotypic antibodies were no longer susceptible to anti-idiotypic suppression (Massey and Kim, 1981). In a subsequent study using BALB/C mice and the PC system, Kim et al., (1983) have reported that anti-idiotypic antibody exerts its inhibitory signal during early B cell triggering. A similar antigen-dependent effect of anti-immunoglobulin sera on the induction of B cell tolerance was observed by others (Pierce et al., 1972; Scott et al., 1977; Ligler et al., 1978).

Our results do not show which population of the spleen cells is affected by in vitro treatment. The fact that the transferred cells can mount a normal secondary anti-DNP response suggests that priming occured. This can be explained by assuming that the in vitro treatment of normal spleen cells with serum containing putative anti-idiotypic antibodies resulted in the masking or blocking of the receptors present on antigen reactive spleen cells. It is quite possible that in this situation the primary response could be weak or delayed (due to the normal immunoglobulin turnover); however, in our experiment, we monitored the response to day 28 after primary immunization and was found negative. Ishizaka and Okudaira (1972) showed that an i.v. injection of mouse anti-OA IgG antibody into DBA/1 mice within 24 hr. after primary immunization with OA prevented both IgE and IgG, antibody responses for a period of over 3 weeks by antibody feedback mechanism. They reported a normal primary anti-OA antibody response 40 days following immunization, when passively administered antibodies were catabolized. Moreover, priming of B cells by anti-idiotypic antibodies, as well as antigen, was reported

by Eichmann and Rajewsky (1975). They found that IgG, fraction of anti-A5A Id antibodies can presensitize the A/J mice for a secondary response challenged by streptococcus A.

It is interesting to note that IgG anti-DNP production was also affected unlike in the in vivo treatments. This can be explained if we assume that the number of IgG B cell carrying the DNP-reactive idiotype, in vitro in a normal spleen culture is quite limited and therefore, more susceptible to the effects of anti-Id antibodies than the large population of DNP-reactive cells available during in vivo study.

We have also observed that when AS obtained from DNP-BP-treated mice was passively transferred into allogeneic DBA/2 ($H\text{-}2^d$) mice prior and shortly after the immunization with DNP-OA, both the primary IgE and IgG antibody responses were suppressed. Furthermore, it was noticed that an identical treatment in the syngeneic system (CBA) did not result in as marked a suppression as obtained with DBA/2. This could be explained by assuming that when DBA/2 mice are immunized with DNP-OA, they express a higher, proportion of anti-DNP antibodies with cross ractive idiotype then CBA mice do. It is known that mice carrying different MHC genes can share the same Igh allotypic markers and therefore, share the idiotypic determinants with the specificity for the same antigen. In addition Ju et al (1978a,b) and Theze and Somme (1979), reported a major set of idiotypes called C-GAT which is present on anti GAT (L-glutamic $acid^{60}$-L-$alanine^{30}$-L-$tyrosine^{10}$) antibodies of all mouse strains, irrespective of Igh allotype markers.

In addition to an Ig-containing fraction, our preliminary data indicated that serum from DNP-BP/DNP-OA mice contains suppressive activity in a fraction that elutes from Sephadex G-100 in a region corresponding to a molecular weight of 10-12,500 daltons (fig. 10). The nature and exact properties of this fraction requires elucidation. Various IgE suppressive factors some of them of low molecular weight, have been reported by several laboratories (Suemura et al, 1977; Kishimoto et al., 1978; Watanabe et al., 1978; Tung et al., 1978; Katz and Tung 1978; Katz, 1979; Katz et al., 1980; Sugimura et al., 1982). None of these, however, was antigen (hapten)-specific and all seemed to be T cell derived. It is interesting to note, however, that recently a soluble factor has been reported by Kresina and Nisonoff (1983) in the serum of anti-P-azophenylarsonate cross-reactive idiotype A (CRIA)-suppressed A/J mice. This factor was not an Ig molecule but upon passive transfer it suppressed the induction of CRIA in adult or neonatal mice. This factor has not been characterized further.

In conclusion our data suggest that serum obtained from DNP-BP-treated-DNP-OA injected mice contains anti-idiotypic antibodies and the mechanism of IgE selective suppression in these mice operates via an idiotypic-anti-idiotypic network as proposed by Jerne (1974). This conclusion is supported by the following observations: a) the serum from DNP-BP/DNP-OA-treated mice effectively suppresses the anti-DNP IgE antibody response upon passive transfer to normal recipients; b) the suppressive effect remains intact after exhaustive absorption of anti-DNP and anti-OA antibodies, and operates across MHC barrier when Igh allotypes are shared between donor and recipient; c) the

suppressive activity of the absorbed serum resides mainly in a fraction eluting from sephadex G-100, with the main bulk of Ig; d) the absorbed suppressive serum contains anti-idiotypic activity, as demonstrated by solid phase RIA inhibition.

These findings perhaps represent the first direct demonstration of T-dependent, antigen-induced auto- anti-idiotypic regulation of the IgE antibody response in mice.

Human immunotherapy of allergic diseases is presently based on the principle of the induction of high levels of IgG blocking antibodies produced in response to repeated injections of the relevant allergen. The present investigation may constitute the basis for explaining the beneficial results of immunotherapy in terms of auto-anti-idiotypic regulation occuring as a result of increase in anti-allergen IgG levels.

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