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Antigen-specific Ige Suppression Induced By Immunization With Dinitrophenyl-bordetella Pertussis Is Mediated Through Interferon-gamma

Michael Hagen

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Antigen-specific IgE suppression induced by immunization with dinitrophenyl-*Bordetella pertussis* is mediated through interferon-gamma.

by

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Abstract

Immunological adjuvants are capable of potentiating immune responses to antigen in a non-specific manner. Adjuvants are, however, selective in their induction of immunoglobulin isotypes. In this regard, numerous studies have demonstrated that the induction of murine IgE responses is particularly sensitive to the nature of the antigen and adjuvant employed. In general, antigen in complete Freund's adjuvant (CFA) suppresses IgE responses, whereas antigen administered with aluminium hydroxide (alum), or with Bordetella pertussis vaccine, leads to enhanced IgE responses to specific antigen.

In the present investigation, immunization of mice with dinitrophenyl-Bordetella pertussis (DNP-BP) failed to induce an IgE response to the hapten (DNP). The immunogenicity of DNP-BP was evident in that anti-DNP IgG antibodies were detected. Pretreatment of mice with DNP-BP determined the formation of anti-DNP IgE B memory cells, since a mixture of DNP-BP- and OA-primed spleen cells was capable of producing high day 7 anti-DNP IgE titers after transfer to syngeneic x-irradiated recipients and booster with 2 μg DNP-OA in alum. Furthermore, mice pretreated with DNP-BP and primed with 2 μg DNP-OA in alum 2 weeks later produced high day 7 anti-DNP IgE levels that subsided to near undetectable levels by day 12-14.

The transient appearance of serum IgE levels was accompanied by normal levels of anti-DNP IgG. The anti-OA response induced as a result of priming with DNP-OA in alum was not affected by the pretreatment with DNP-BP. In the search for a mechanism responsible for the observed isotype and antigen-specific regulation, suppressor cells and regulation through the induction of auto-anti-idiotypic antibodies was ruled out. IgG subclass analysis revealed however, that mice pretreated with DNP-BP had elevated levels of IgG2a and reduced levels of IgG1, as compared to control mice. This led to an investigation of the role of lymphokines in DNP-BP-pretreated mice.

Significant levels of interferon-gamma (IFN-gamma) were detected in the supernatants of spleen cell cultures obtained from mice pretreated with DNP-BP. Moreover, treatment of mice with a monoclonal antibody to IFN-gamma shortly after immunization with DNP-BP, not only reduced anti-DNP IgG2a levels, but prevented the decline of the anti-DNP IgE antibodies induced by priming with DNP-OA in alum.

These results demonstrate that DNP-BP-induced IFN-gamma production can modulate immunoglobulin isotype expression <u>in vivo</u> in an antigen-specific manner.

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ABBREVIATIONS

alum	$Al(OH)_3$
ADS	serum depleted of anti-DNP antibody
BA	*Brucella abortus*
BF	binding factor (ie. IgE binding factors)
BP	*Bordetella pertussis*
BSA	bovine serum albumin
BSS	Hanks' balanced salt solution
CFA	complete Freund's adjuvant
cpm	counts per minute
DMEM	Dulbeccos' Modified Eagles' Medium
DNP	dinitrophenyl
DNP-BP	dinitrophenyl-*Bordetella pertussis*
EIA	enzyme linked immunosorbent assay
FCS	fetal calf serum
GA	glutaraldehyde fixed
Id	idiotype
IFA	incomplete Freund's adjuvant
IFN	interferon
IL	interleukin (ie. interleukin 4)
i.p.	intraperitoneal
i.v.	intravenous
KLH	Keyhole limpet hemocyanin
Lys	lysine
mAb	monoclonal antibody
MLN	messenteric lymph node

ABBREVIATIONS CONTINUED

NAS	non-absorbed serum
Nb	Nippostrongylus brasiliensis
NIP	4-hydroxy-3-nitro-5-iodo-phenylacetylsuccinamide
NMS	normal mouse serum
OA	ovalbumin
OU	optical density units
PBS	phosphate buffered salt solution
PCA	passive cutaneous anaphylaxis
rIFN	recombinant interferon
RIA	radioimmunoassay
SA-AP	streptavidin-alkaline phosphatase
Seph.	Sepharose
T_H1	helper T lymphocyte, subpopulation 1
T_H2	helper T lymphocyte, subpopulation 2
T_s	suppressor T lymphocyte
TNP	trinitrophenyl
TNP-KLH	trinitrophenyl Keyhole limpet hemocyanin
μg	microgram
μl	microliter
VSV	Vesicular stomatitis virus

1. Introduction and Historical Review

Although the origins of the immune system remain unclear, it is generally conceded that it developed with the appearance of Eukaryotic organisms from a necessity to discriminate between self and non-self (Cooper, 1976; Marchalonis, 1977). This discrimination, although generally beneficial to the organism, must be highly regulated in order not to mediate negative effects on the host organism. In man, where the development of the immune system has reached its peak, a number of immunoregulatory deficiencies exist that threaten the life, or well being, of the individual affected. Indeed, even normal immune responsiveness to foreign determinants such as allergens, can be detrimental to a large segment of the population prone to the development of allergies.

It is hoped that through the understanding of the basic mechanisms of immune responsiveness and regulation, one can gain insight into mechanisms of controlling these undesirable effects.

It is generally accepted that the generation of immune responses requires the interaction of a variety of distinct immunocompetent cell types. Since the initial descriptions of cellular interactions in response to immunization (Claman et al., 1966; Miller and Mitchell, 1968), numerous studies have demonstrated that antibody formation is the end result of the cooperation of T and B lymphocytes, as well as a

variety of antigen presenting cells, including B cells (Mitchison, 1971; Katz and Benacerraf, 1975; Unanue, 1984; Lanzavecchia, 1985). In order for an immune response to occur, T cells must recognize antigen in the context of cell surface glycoproteins encoded by genes of the major histocompatibility complex (MHC)(Rosenthal, 1978; Unanue, 1981). The exact ways in which these cells collaborate in an exquisitely coordinated and regulated fashion is still unclear. However, communication between cells recognizing and responding to antigen is known to involve cognate and non-cognate interactions, as well as a number of soluble molecules, including lymphokines. It is these intercellular messages that dictate cellular expansion and proliferation in response to antigenic stimuli (Howard and Paul, 1983; Singer and Hodes, 1983; Hamaoka and Ono, 1986).

The IgE response shares many mechanisms with other immunoglobulin isotypes in terms of its induction by protein antigens. It has however, several unique characteristics that are not easily demonstrable in IgG and IgM responses to antigen (Ishizaka, 1984). The importance of understanding the mechanisms that regulate IgE production is of considerable importance because of the role that IgE plays in the mediation of human allergic disorders.

Attempts to understand the regulation of IgE synthesis began with the observations that IgE was a distinct class of skin-sensitizing antibody (Ishizaka et al., 1966). Since then, its immunochemical characterization (Johansson and

Bennich, 1967), unique sensitivity to induction by certain adjuvants, and dependence for induction on certain animal strains (Revoltella and Ovary, 1969; Levine and Vaz, 1970), has led to the development of animal prototypes as models for human atopic disease.

1.1 Animal models and adjuvants for the study of IgE.

Although reaginic antibody formation has been demonstrated in rats immunized with antigen in association with Bordetella pertussis (BP)(Binaghi et al., 1964; Mota, 1964), it was not until the studies of Revoltella and Ovary (1969), and Levine and Vaz (1970), that a persistent and boosterable antigen-specific IgE response could be reproducibly induced in mice. Those studies found that mice fell into two distinct response patterns (low and high), and that the induction of IgE depended upon a number of factors.

The basis for the distinction between responder and non-responder strains of mice was shown to be genetically controlled, and linked to a number of genes both within and outside the MHC (H-2 complex)(reviewed by Lehrer and Bozelka, 1982). In mice, antigen-specific IgE was induced when minute doses of antigen were administered with appropriate adjuvants (Clausen et al., 1969; 1970). The importance of adjuvants for the induction of IgE was subsequently confirmed by numerous studies, and immunization of rodents without adjuvant consistently failed to induce IgE antibody (reviewed by Ishizaka, 1976).

The results of these and other studies demonstrated that the adjuvants of choice for the induction of antigen-specific persistent IgE responses include B. pertussis vaccine and its extracts, and $Al(OH)_3$ (alum)(Mota, 1967; Clausen et al., 1969; Revoltella and Ovary, 1969; Levine and Vaz, 1970). On the other hand, the administration of complete Freund's adjuvant (CFA), or incomplete Freund's adjuvant (IFA), with antigen is ineffective at eliciting IgE responses (Mota, 1964; Katz, 1978; Clausen et al., 1969; Tada, 1975). Those studies did however establish that all of the above adjuvants were capable of inducing good IgG responses against soluble antigens.

1.2 T-cell dependence of the IgE response.

The dependence of the IgE response on the presence of T cells was established from observations that nude mice (Michael and Bernstein, 1973) and neonatally thymectomized rats (Okumura and Tada, 1971), were incapable of producing IgE. Subsequent studies found that although incapable of antibody production, nude mice developed significant numbers of IgE bearing B cells in their spleens and lymph nodes in response to both T-dependent and T-independent antigens (Okudaira et al., 1980).

In a separate study, it was observed that a substantial number of IgE-bearing B cells were present in the spleens and lymph nodes of neonatally thymectomized rats following infection with Nippostrongylus brasiliensis (Urban et al.,

1977). These results suggested that IgE B cells could proliferate in response to antigen in the absence of T cell help. However, their differentiation into IgE-secreting plasma cells appeared to be strictly T-cell dependent (Urban et al., 1977; Okudaira et al., 1980).

Effective collaboration between T helper (T_H) cells and B cells was eloquently demonstrated in studies using hapten-carrier conjugates as immunogens (Rajewski et al., 1969; Mitchison, 1969; 1971). It was demonstrated that T cells recognized carrier determinants, whereas B cells responded to haptenic determinants. This collaboration, known as the "carrier" effect, was soon established for the IgE response as well (Okudaira and Ishizaka, 1973).

More recently, the collaboration between carrier-primed T cells and hapten-primed B cells has been demonstrated by adoptive transfer (Hamaoka et al., 1973; 1974), and in in vitro cultures (Kishimoto and Ishizaka, 1973). The similarity between IgE and IgG responses in their requirement for T-cell help, does not necessarily mean however that helper T cell populations are the same for both isotypes. Indeed, the results of numerous studies, in both mice and rabbits, has demonstrated the existence of discrete isotype-selective helper T cell populations (Kishimoto and Ishizaka, 1973a; 1973b; Hamoaka et al., 1974; Kojima and Ovary, 1975).

Although the IgE response is strictly thymus-dependent, a number of experimental manipulations leading to partial T-cell depletion have resulted in enhanced IgE production. The exposure to low doses of irradiation (Tada, 1978; Katz, 1980), or treatment with cyclophosphamide (Katz, 1980), allowed the development of persistent IgE responses in rats characteristically displaying low, unboosterable IgE titers. Indeed, it has been recently reported that treatment with Cyclosporin A at the time of exposure to conventional protein antigens potentiates antigen-specific IgE responses (Chen, 1988).

Collectively, the results described above led to the conclusion that the IgE response is normally regulated by isotype selective T suppressor (T_s) cells (Hirashima et al., 1980; Suemura et al., 1980; Kawanishi et al., 1983; Jacobs et al., 1986).

1.3 Antigen-specific regulation of IgE responses.

The results of numerous studies over the past 20 years have clearly demonstrated that the IgE response is regulated by both antigen-specific and non-specific mechanisms. Attempts to regulate the IgE response in an antigen-specific manner have generally focused on three areas; 1) the tolerization of B_ϵ cells; 2) the manipulation of T cells capable of modulating the proliferation and differentiation of B_ϵ cells; 3) the induction of anti-idiotypic antibodies.

A number of methods have been successful at the induction of tolerance in both naive and previously sensitized animals. Tolerance can be induced through the administration of hapten-coupled copolymers of D-glutamic acid-D-lysine (dGL), or to polysaccharides (Katz et al., 1973; Watanabe et al., 1977). Thus, administration of dinitrophenylated derivatives of dGL prior to immunization with DNP-ovalbumin (OA) was capable of suppressing primary and secondary IgE anti-hapten antibody responses. Furthermore, injection of dGL into immunized animals terminated an ongoing anti-hapten response (Katz et al., 1973). The termination of anti-hapten antibody production was however isotype non-selective, in that IgG and IgM responses were also affected by this treatment. These results were due to the inactivation of hapten-specific B cells by treatment with dGL in the absence of T_s cells (Katz et al., 1973).

In a subsequent investigation, it was demonstrated that the administration of DNP-polyvinyl alcohol (PVA) was also capable of inactivating DNP-specific B_ϵ cells (Lee and Sehon, 1981). Other methods successful at the induction of tolerance include the repeated feeding of protein antigens (Vaz et al., 1977) and the injection of antigen covalently coupled to non-immunogenic carriers such as isologous gamma globulin (Filion et al., 1980).

The induction of antigen-specific IgE suppression through the manipulation of T cells has been reported by

several laboratories. Generally, these mechanisms operate through the induction of suppressor T cell populations, however, carrier-specific T-cell tolerance in the absence of detectable T_s cells has been reported (Colby and Strejan, 1980).

The induction of T_s cells is based on the fact that the antigenic determinants recognized by T cells may not necessarily be the same as those recognized by B cells (Ishizaka et al., 1975; Takatsu and Ishizaka, 1975). It has been demonstrated that antigen-specific T_s cells can be induced through the use of urea-denatured (Ishizaka et al., 1975; Takatsu and Ishizaka, 1975; 1976), glutaraldehyde polymerized (HayGlass and Strejan, 1985), or polyethylene glycol (PEG) conjugated antigens (Lee and Sehon, 1978; Lee et al., 1981) administered in the absence of alum as an adjuvant. The treatment of mice with these chemically modified antigens was capable of suppressing primary IgE and IgG responses when administered at the time of, or shortly after, antigenic stimulation.

Another approach to the regulation of antigen-specific IgE responses has been through the induction and administration of anti-idiotypic antibodies (Blaser et al., 1980; Wetterwald et al., 1986). The basis of this immunoregulatory approach is that anti-hapten antibodies may be restricted to certain predominant idiotypes. Therefore, the manipulation of a particular idiotype by an anti-idiotypic antibody could suppress the development of an

antibody response. This approach seems to be particularly well suited to the IgE response, since it has been demonstrated that IgE is more susceptible to anti-idiotypic regulation than IgG (Blaser et al., 1980). Both a primary and ongoing IgE antibody response to benzyl-penicilloyl could be suppressed by the passive administration of isologous anti-idiotypic antibodies (Blaser et al., 1980; Blaser and de Weck, 1982). The significance of these and other studies remains questionable however, as it is known that the idiotypic repertoire to most antigens, including small haptenic molecules, is heterogeneous (Askonas et al., 1970). Furthermore, it has also been demonstrated that the suppression of an idiotypic determinant by anti-idiotypic antibodies generally involves a shift in the idiotypic repertoire, and not a decrease in total antibody production (Kelso et al., 1980; Reth et al., 1981; Kim, 1982).

1.4 Isotype-specific regulation of IgE: T cells and their factors.

Experimental data accumulated over the past 20 years have provided a considerable amount of evidence suggesting that differences in the regulation of IgG and IgE responses to antigens may be due to distinct isotype-specific T_H and T_S cell populations (reviewed in Ishizaka, 1984).

IgE-selective antigen non-specific T suppressor cells were initially demonstrated in SJL mice by Watanabe et al. (1976; 1977) and Chiorazzi et al. (1976). It was shown that

increases in IgE associated with low-doses of irradiation could be effectively cancelled by the adoptive transfer of syngeneic Lyt-1$^+$ T cells (Watanabe et al., 1976; 1977; Chiorazzi et al., 1976; Katz, 1980). Subsequently, it was demonstrated that suppressive factors could be isolated from these cells. In another study, it was observed that the administration to mice, of hapten (DNP)-coupled Mycobacterium tuberculosis (Myc), suppressed the development of anti-hapten IgE responses when challenged with alum-absorbed DNP-OA (Kishimoto et al., 1976). The results of that and subsequent studies revealed that T_s cells were generated in the spleens of DNP-Myc-primed mice, and that these cells were capable of releasing an antigen non-specific suppressor factor (Seumura et al., 1977) More recently, similar results were obtained using phosphorylcholine conjugated mycobacterium (Kishimoto et al., 1981). The results of those studies clearly showed that although hapten-specificity determined the induction of the suppressive molecules, the effect of these molecules was antigen-non-specific (Kishimoto et al., 1976; Seumura et al., 1977; Kishimoto et al., 1981).

The immunosuppressive nature of mycobacterium conjugates, through the induction of antigen-non-specific T-cell factors, was supported by separate studies showing that IgE-selective T_s cell activity was enhanced in mice injected with CFA (Tung et al., 1978). It should be noted that Mycobacterium is an essential component of CFA (Freund et

al., 1937). The repeated administration of CFA induced the selective suppression of IgE to unrelated antigens (Tung et al., 1978). It was found that CFA induced T_s cells, and that the serum of CFA-treated mice contained a soluble factor that was capable of suppressing the irradiation-enhanced IgE responses of SJL mice (Tung et al., 1978).

The same group of investigators subsequently detected in the serum of CFA-treated mice, a molecule capable of potentiating IgE responses (Katz et al., 1979). Indeed, both suppressive, and enhancing activities could be detected in the same serum. These two activities could be separated from each other due to the affinity of the enhancing factor to lentil lectin (Katz et al., 1979). In a series of recent publications, it was proposed that both the suppressive and enhancing factors are part of a regulatory cascade of molecules that is triggered by the binding of IgE to the Fc_ϵ receptor on B cells (Marcelletti and Katz, 1984a; 1984b; 1984c). The regulation of the IgE response by these factors is thought to be due to the relative ratio of suppressive factor to enhancing factor.

A review of the literature pertaining to the regulation of IgE in the last 10 years, reveals that this field has been dominated by the discovery of soluble T cell-derived molecules that have specificity for the constant heavy chain region (Fc) of IgE (Ishizaka 1984; Ishizaka, 1988). These molecules were initially described in a study examining the effects of non-specific stimulation on IgE production in

vitro (Suemura and Ishizaka, 1979). That study was initiated based on previous observations that parasitic infections potentiated IgE in rodents. It is well documented that 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), are very potent inducers of IgE. It was observed (Suemura and Ishizaka, 1979) that mesenteric lymph node (MLN) cells from rats primed with DNP-OA in alum developed selectively-enhanced IgE responses when cultured with DNP-OA in the presence of T cells from mice infected with Nb. IgG production was not affected in this system.

Soon thereafter, these investigators isolated an IgE potentiating-factor from T cells of rats infected with Nb. (Suemura et al., 1980; Yodoi et al., 1980). A detailed analysis revealed that this factor was the product of Fc_{ϵ} receptor positive T cells, and affected IgE bearing B cells (Suemura et al., 1980; Yodoi et al., 1980). Subsequently, it was shown that IgE-binding factors exist in two distinct forms, and that they are distinguishable on the basis of carbohydrate content, differential affinity for lectins, and IgE regulatory activity. IgE binding factors (IgE-BF) could be isolated from MLN and spleen cells of rats treated with either CFA or BP vaccine (Hirashima et al., 1980; 1981a; 1981b). It was determined that factors induced as a result of treatment with BP or Nb had potentiating properties

(Yodoi and Ishizaka, 1980; Hirashima et al., 1981a), whereas the factors isolated from culture or serum from rats treated with CFA were suppressive (Hirashima et al., 1980).

The functional roles of these molecules were evident from studies showing that immunization with antigen using alum, or BP as adjuvants induced IgE-potentiating factors and good IgE levels, whereas immunization using antigen in association with CFA induced IgE-suppressive factors and failed to induce IgE antibody production (Uede et al., 1982; Jardieu et al., 1984). A comprehensive review of the intricacies of IgE-BF induction, regulation, and expression has recently been published by Ishizaka (1988).

The T cell-derived IgE-BF's are glycoproteins that are apparently heterogeneous in their molecular weight (Uede et al., 1983; Jardieu et al., 1985). The cloning of a rat IgE-BF gene, and its subsequent amino acid sequencing, indicated that the molecular weight heterogeneity is probably due to the cleavage of a larger common precursor molecule into many smaller molecules (Jardieu et al., 1985). Indeed, the results of extensive molecular and physio-chemical characterization indicate that both IgE-potentiating and suppressive molecules share a common structural gene (Martens et al., 1985; 1987). Through the use of glycosylation inhibitors such as tunicamycin, these investigators were successful in switching production from IgE-potentiating factor to IgE-suppressive factor.

The type of IgE-BF formed seems to depend upon two distinct inducer molecules: a glycosylation enhancing factor (GEF), and a glycosylation inhibiting factor (GIF). It has been shown that these two molecules regulate the post-transcriptional glycosylation of IgE-BF (reviewed by Ishizaka, 1988). These molecules are the products of separate T lymphocytes, GIF being produced by Lyt-2$^+$ cells, GEF by Lyt-1$^+$ cells. It is proposed that these two molecules compete with each other with respect to the glycosylation of IgE-BF, and that the balance between these two molecules determines the nature of the IgE-BF produced, and hence, the magnitude of the ensuing IgE response (Iwata et al., 1984).

The involvement of these factors in the immunoregulation of human allergic disorders is implicated by recent studies that have isolated these molecules from the T cells of patients with hyper IgE syndrome (Saryan et al., 1983), from human hybridoma cell lines (Huff et al., 1986), and from the sera of normal individuals (Leung et al., 1984).

1.5 Reciprocal regulation of isotype expression by lymphokines.

Since initial investigations demonstrated that soluble products were released from T cells as a result of antigenic stimulation, and that these were involved in driving B cells to immunoglobulin secretion, (Dutton et al., 1971: Waldman

and Munro, 1973), a number of technical difficulties have delayed the understanding of the mechanisms involved. It was not until the development of monoclonal antibodies to individual lymphokines, the availability of recombinant lymphokines, and the generation of T helper (T_H) cell clones, that a clearer understanding of their roles in immunoregulation became possible.

Recent studies have shown that T_H cells, as a result of antigen presentation in association with self MHC, are capable of releasing lymphokines that transmit regulatory signals to B cells. The results of these studies have demonstrated that immunoglobulin isotype expression is regulated by many of the same lymphokines that control B cell activation, growth, and differentiation (reviewed by Coffman et al., 1988). The generation of T_H cell clones, has led to the separation of T helper cells into two separate subtypes of $CD4^+$ ($Lyt\text{-}1^+$ $L3T4^+$ $Lyt\text{-}2^-$) lymphocytes (Kim et al., 1985; Mosmann et al., 1986).

These two T_H cell types have been designated as T_H1 and T_H2, based on their production of lymphokines, and the type of help that they deliver to B cells (Mosmann et al., 1986). Antigen, or concanavalin-A (Con-A), stimulates T_H1 cells to synthesize a number of lymphokines including interleukin-2 (IL-2), IFN-gamma, and lymphotoxin (but not IL-4). T_H2 cells on the other hand, produce IL-4 and IL-5, but not IFN-gamma (Mosmann et al., 1986; Mosmann and Coffman, 1987). Although both T_H1 and T_H2 cells are capable of providing

help to B cells in the induction of an antibody response, several differences exist that affect the expression of immunoglobulin isotype.

In examining the regulation of isotype expression in bacterial lipopolysaccharide (LPS)-stimulated B cells, it was observed that the supernatants of T_H2 clones caused a significant increase in the production of IgG1 (Isakson et al., 1982; Bergstedt-Lindquist et al., 1984), IgE and IgA (Coffman and Carty 1986). IgG1 production was increased 5-20 fold, whereas IgE levels were elevated from below threshold to 100-1000 fold above threshold (Coffman and Carty, 1986). The increased levels of both IgG1 and IgE production were the result of IL-4 activity in the T_H2 supernatants. The effect was blocked by anti-IL-4 antibody (Vitetta et al., 1985; Coffman et al., 1986).

Other studies have also shown that IL-4 determined a preferential synthesis of IgG1 and IgE (Isakson et al., 1982; Paul and Ohara, 1987), and that it inhibited the secretion of IgM, IgG2b, IgG3, and IgG2a (Coffman and Carty, 1986; Snapper and Paul, 1987). In terms of a mechanism of action, IL-4 was shown to inhibit the germ line transcription of IgG2b in spleen cells cultured in vitro with LPS (Lutzker, 1988). This led to the suggestion that lymphokines regulated isotype switching by altering the accessibility of specific C_H loci to a common switch-recombination mechanism.

T_H1 cell supernatants are poor stimulators of immunoglobulin production as compared to T_H2 cell supernatants. They have been shown, however, to be capable of eliciting antigen-specific responses from hapten-primed B cells in culture (Giedlin et al., 1986). Indeed, controversy exists as to whether or not T_H1 cells should be classified as helper cells at all (Killar et al., 1987; Boom et al., 1988). Although T_H1 cells are not as efficient in the induction of immunoglobulin production as T_H2, they are just as capable of inducing B cell proliferation as T_H2 cells. This has led to the suggestion that T_H1 clones might produce molecules that inhibit in vitro immunoglobulin production (Coffman et al., 1988). Indeed, IFN-gamma, a T_H1 cell product, is inhibitory at high concentrations, and the inclusion of a monoclonal anti-IFN-gamma antibody to culture has been reported to enhance responses (Coffman et al., 1988). It has been suggested that the poor inductive capacities of T_H1 cell supernatants in vitro may represent an artefact of culture, rather than an accurate reflection of the in vivo activity of T_H1 cells, and that the failure of T_H1 cells to induce B cell differentiation might be due to insufficient production of IL-2 (Coffman et al., 1988; Mosmann and Coffman, 1989).

In experiments where the same B cell preparations were stimulated by both T_H1 and T_H2 clones, T_H1 clones were found to induce a 20-50 fold increase in the levels of IgG2a production versus that induced by T_H2 clones (Coffman et al.,

1988). The results of these studies have been confirmed by Snapper and Paul (1987), who reported that IFN-gamma induced IgG2a production in resting, surface IgG^{-ve} B cells, upon its removal prior to the addition of LPS, or of LPS plus anti-IFN-gamma antibody (Snapper et al., 1988b).

Collectively, the above experiments suggest that IL-4 promotes the expression of IgG1 and IgE, whereas IFN-gamma selectively induces IgG2a production in B cells initially only expressing surface IgM (Snapper et al., 1988a; 1988b). These molecules have also been shown to be mutually antagonistic. IFN-gamma inhibits IL-4-dependent IgG1 and IgE responses (Coffman and Carty, 1986), whereas the IgG2a enhancing activities of IFN-gamma are negated by the presence of IL-4 (Snapper et al., 1988a). Although both IL-4 and IFN-gamma have recently been implicated in the modulation of Fc_{ϵ} receptors on the surface of murine B cells (Conrad et al., 1987), their relationship to IgE-BF is unclear.

Although the aforementioned studies were all based on the in vitro effects of T_H cell subpopulations and their lymphokines, a large body of data has been generated implying an immuno-regulatory role for lymphokines in vivo. The injection of anti-IgD antibodies into mice, results in the polyclonal activation of both T and B cells, and a selective increase in non-specific serum levels of IgG1 and IgE (Finkelman et al., 1987). It has also been demonstrated that T cells from anti-IgD-treated mice secrete elevated

levels of IL-4, and that the administration of an anti-IL-4 antibody to mice treated with anti-IgD abrogates the enhancement of IgE (Finkelman et al., 1986). In that study, it was also shown that the administration to mice of IFN-gamma after treatment with anti-IgD dramatically suppressed the IgG1 and IgE levels. Furthermore, the administration of an anti-IFN-gamma monoclonal antibody together with anti-IgD resulted in increased levels of IgE and IgG1 production (Finkelman et al., 1986).

On the other hand, Brucella abortus (BA) vaccine stimulated the production of IgG2a and IFN-gamma (Finkelman et al., 1988). Indeed, the administration of BA together with anti-IgD resulted in decreased production of IgG1 and IgE (Finkelman et al., 1988).

The effects of T_H cell lymphokines are not restricted to B lymphocytes. Recent studies showed that IL-2 (T_H1) and IL-4 (T_H2) also act as T cell growth factors (Bottomly, 1988; Fernadez-Botran, 1988), and that IFN-gamma inhibits T_H2 cell proliferation induced as a result of exposure to antigen (Gajewski and Fitch, 1988).

These observations suggest that the T_H cell populations contain mutually regulatory subsets, and that the isotypic profile of an immune response to an antigen depends on the relative ratios of T_H1 to T_H2 cells, and their lymphokines, in vivo. This expression of antibody isotypes may have profound effects on the nature of the immune response.

The present investigation was based upon the initial

observations of a previous study that described the suppression of an anti-DNP IgE response induced by the pretreatment of mice with dinitrophenyl-*Bordetella pertussis* prior to immunization with dinitrophenyl-ovalbumin. The purpose of this study was to discern the mechanism responsible for the observed antigen-specific and isotype-selective suppression. As the IgE response is sensitive to a number of immunoregulatory mechanisms, we attempted to demonstrate whether the observed responses were the result of the induction of suppressor T cells, auto-anti-idiotypic antibody production, an antibody feedback mechanism, or through the differential induction of lymphokines.

The results will demonstrate that the *in vivo* production of immunoglobulin isotypes can be modulated by conventional antigens, through the preferential induction of lymphokines. Specifically, immunization with DNP-BP determines an increase in the levels of IFN-gamma as detected by both *in vitro* and *in vivo* experimentation. The results will demonstrate that the antigen-specific immunoglobulin isotype profile is influenced directly through elevated levels of IFN-gamma.

2. Materials and Methods

2.1 Animals.

Balb/c and CBA mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in our animal facility by brother/sister matings. All mice used in these studies were between 8-12 weeks of age. Sprague-Dawley female rats (retired breeders) were obtained from Charles River Canada (Ottawa, Ontario).

2.2 Antigens, Lymphokines and Antibodies.

Bovine serum albumin (BSA) fraction V, was purchased from Sigma Chemical Co. (St. Louis, MO), crystallized ovalbumin (OA) was from Miles Laboratories (Rexdale, Ontario), and keyhole limpet hemocyanin (KLH) was fr m Biomarine Pacific Supply Co. (Venice, CA). 2,4-dinitrobenzene sulfonic acid, sodium salt (Eastman-Kodak Co., Rochester, NY) was coupled to proteins as previously described (Colby and Strejan, 1980). DNP_{22}-BSA and DNP_{5}-OA were stored at -20°C. TNP_{53}-KLH was stored at 4°C. The subscripts refer to the average number of mols DNP/mol of carrier, assuming a molecular weight of 40,000 for OA, 72,000 for BSA and 800,000 for the dissociated form of KLH. NE-2,4-DNP-L-lysine (DNP-Lys) and N-2,4-DNP-glycine (DNP-Gly)(Sigma) were stored dessicated and protected from light at -20°C.

NIP_{10}-OA and NIP_{22}-BSA were pr pared according to the procedures described by Weinberger et al. (1979, 1980).

Briefly, 1 mg 4-hydroxy-3-nitro-5-iodo-phenylacetyl-succinamide (NIP)(Biosearch Labs, San Rafael, CA) was coupled to 5 mg protein. The degree of conjugation was estimated based on a molar extinction coefficient of 5000 at 430 nm (Brownstone and Mitchison, 1966). NIP_{10}-DNP_3-OA was prepared as described above by the addition of NIP to DNP_3-OA. The degree of haptenation was corrected for optical density reading overlaps at 360 nm and 430 nm.

Bordetella pertussis vaccine containing 20 OU/ml (U.S. Standard) was obtained from the Armand Frappier Institute (Laval, Quebec). Ascaris suum antigen was prepared in this laboratory as previously described (Hussain et al., 1973). 2,4-Dinitrophenyl (DNP)-BP was prepared as described for Mycobacterium tuberculosis (Kishimoto et al., 1976). Briefly, BP vaccine containing $4.5x10^{11}$ killed organisms was centrifuged at 10,000xg for 20 min. in a refrigerated centrifuge, the pellet was resuspended in isotonic saline and the cells were washed by centrifugation 5 times. The washed organisms were then resuspended in 0.25 ml 0.1M Na_2CO_3 to which were added 2.5 µl 1-Fluoro-2,4-dinitrobenzene (DNFB)(Eastman-Kodak Co.). The mixture was then gently stirred at 37°C for 60 min., protected from light. The cells were then washed to remove unreacted hapten and suspended in borate-buffered saline pH 8.4. DNP-BP were stored at 4°C at a concentration of $2x10^{10}$ organisms/ml. The number of DNP groups was determined by lysing the cells in boiling 1N NaOH and determining the

absorbance at 360 nm. Assuming a molar extinction coefficient of 17,500, there were 5-20x10^6 DNP groups/cell, depending on the batch conjugated. Preparation of DNP-BP for cell cultures was done under aseptic conditions.

NIP-BP was prepared essentially as described for DNP-BP. Briefly, 9x10^{10} BP organisms were coupled with 2.64 milligrams NIP. This procedure generally yielded 2-3x10^7 NIP groups per cell as determined spectrophotometrically at 430 nm.

Purified murine recombinant interferon-gamma (rIFN-gamma) and affinity purified rat IgG1 monoclonal antibody (mAb) specific for rIFN-gamma (XMG1.2) were a generous gift of Dr. R. L. Coffman (DNAX, Palo Alto, CA). Affinity purified rat IgG1 mAb specific for IFN-gamma (XMG6) and for 4-hydroxy-3-nitrophenylacetyl (NP)(J4.1) were kindly provided by Dr. F. Finkelman (USUHS, Bethesda, MD).

2.3 Myelomas and Hybridomas.

The mouse anti-DNP myeloma cell lines MOPC-315 and MOPC-460 (IgA, kappa) were kindly supplied by Dr. M. Potter (NCI, Bethesda, MD). The cells were thawed from dry ice immediately upon arrival and were injected into the intraperitoneal (i.p.) cavity of BALB/c female mice primed with tetramethylpentadecane (pristane)(Pfaltz and Bauer, Stamford, CT) 7-14 days earlier. After the development of ascites (usually 11-14 days after inoculation), the abdominal cavity of mice was drained using an 18 G 1 inch

needle at 3-4 day intervals. The ascites were collected and stored at -20°C until needed.

The following hybridomas were constructed as described below: 3/B9, 1/D8, 1/C12, 1/F2, D12, (anti-DNP IgE); AC2D12, AC6/93 (anti-DNP IgG1); B4D4, C1E10 (anti-DNP IgG2a); D2.1/E, F3.2/E (anti-NIP IgE); anti-D12 (anti-idiotype). TIB-142 (anti-DNP IgE, kappa)(Rudolph et al., 1981) was purchased from the American Type Culture Collection (ATCC, Rockville, MD).

i) Cell preparation and feeders.

Hybridomas were constructed according to a modified version of the procedure described by Fazekas de St. Groth and Scheidegger (1980). Individual wells of a 96-well flat-bottomed tissue culture plate (Costar, Cambridge, MA) were pre-incubated with peritoneal exudate cells (PEC) obtained from normal syngeneic mice. Briefly, mice were sacrificed by cervical dislocation prior to peritoneal infusion of 6 ml ice-cold 0.34M sucrose solution. After abdominal massage, the cells were collected and washed with Hanks' balanced salt solution (BSS)(GIBCO, Burlington, Ontario) treated with 0.85% ammonium chloride if necessary, washed again and suspended in Dulbecco's Modified Eagle's Medium (DMEM)(GIBCO), supplemented with 20% fetal calf serum (FCS), 10% NCTC-109 (GIBCO), 1% non-essential amino acids, 1% Na-pyruvate, 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml fungizone, 1.6×10^{-5}M thymidine, 10^{-4}M hypoxanthine, and 4×10^{-7}M aminopterin (HAT). Each

well received $2x10^4$ PEC in 100 µl, 2-3 hours prior to fusion. The plates were incubated at 37°C in a 5% CO_2 incubator until needed.

ii) Fusion.

For fusion, the non-secreting myeloma cell line SP2/0-Ag14 (ATCC) was expanded and maintained in log phase growth in 25 cm^2 flasks (GIBCO) in DMEM-10% FCS. The thioguanine-sensitivity of the myeloma was ensured by passage through medium containing $2x10^{-5}$M 6-thioguanine (Sigma) every 4-6 weeks. Erythrocyte-free single spleen cell suspensions from appropriately immunized mice were prepared as described below.

Spleen cells for anti-DNP IgG1 antibodies were obtained from CBA mice injected with 10^9 DNP-BP i.p. 14 days prior to immunization with 2 µg DNP-OA in alum i.p. Fusion was performed on day 7 relative to the priming. For anti-DNP IgG2a antibodies, Balb/c mice were primed with $2X10^9$ (100 µg) DNP-BP in complete Freund's adjuvant (CFA)(Difco Laboratories, Detroit, MI) 28 days before fusion, and boosted with the same amount of antigen in saline intravenously (i.v.) 3 days before fusion.

For anti-DNP IgE, Balb/c mice were sublethally irradiated (660 R) prior to receiving a mixture of spleen cells from pretreated mice 7 days prior to fusion. One group of donor mice was primed with 0.5 µg OA in alum 14 days prior to fusion, the other with 10^9 DNP-BP on days -23, -21, and -19 relative to fusion. After reconstitution with

$20X10^6$ cells from each donor group, recipient mice were immunized with 2 μg DNP-OA in alum. The anti-DNP IgE PCA titer of the recipient mice on the day of fusion was 1:1280.

For the production of an anti-idiotypic antibody, mice were repeatedly immunized with 100 μg affinity-purified anti-DNP IgE D12, crosslinked with glutaraldehyde as described below. Mice were boosted i.v. 3 days prior to fusion, after significant levels of anti-D12 antibodies were detected in their serum by enzyme immunoassay (EIA) (titer $>10^5$).

The myeloma and spleen cells were suspended in serum-free DMEM at a ratio of 5:1 (spleen cells to myeloma). They were pelleted in a 15 ml conical centrifuge tube at 400xg for 5 minutes. The pellet was then overlayed with 0.2 ml DMEM at 37°C. Fusion was accomplished by the addition of 0.8 ml 50% polyethylene glycol (PEG 1000)(BDH, Toronto) with stirring, to the cell pellet over a period of one minute (final PEG concentration 40%). The mixture was stirred for an additional minute prior to the addition of 1 ml of warm (37°C) DMEM. At the end of 3 minutes from the onset of fusion, the cells were pelleted by centrifugation at 500xg for 3 minutes. The PEG-DMEM was then decanted and 5 ml warm DMEM was added to disperse the cell pellet. The cells were centrifuged at 400xg for 5 minutes, the supernatant was decanted, and 10 ml DMEM-HAT was added gently to the pellet so as not to disrupt it. The cells were placed at 37°C for 7 minutes. The pelleted cells were dispersed by one pipette

aspiration and discharge cycle, and suspended in a volume such that $3x10^5$ cells would be added to each well.

iii) Screening.

Plates were examined for growth on day 7 after fusion, and fed by replacement of 100 µl HAT. Antibody production was usually measurable in actively growing wells by day 11. Hybridomas were cloned twice by limiting dilution and expanded into 24-well plates.

iv) Cell freezing and reconstitution.

For long term storage, antibody-producing clones were washed and suspended to an approximate density of $5x10^6$-10^7 cells/ml in 1 ml of DMEM supplemented with 10% FCS, non-essential amino acids, L-glutamine, Na-pyruvate, penicillin, streptomycin, fungizone (complete medium), and 10% dimethyl sulphoxide (DMSO) (ATCC). The cells were frozen at -70°C overnight, and then transferred to liquid nitrogen until needed. Cells recovered from storage were rapidly thawed at 37°C, immediately placed into 6 ml of DMEM complete medium, and incubated at 37°C in a 25 cm^2 tissue culture flask (Nunc-GIBCO, Burlington, Ontario). The next day, half of the medium was replaced with fresh medium to dilute out the DMSO. Alternatively, rapidly thawed cells were injected into the peritoneal cavity of mice injected 7-28 days previously with pristane.

2.4 Cell preparation and culture.

Mouse spleens were removed aseptically and single cell suspensions were prepared in BSS. The cells were then washed by centrifugation and treated with 0.85% ammonium chloride to remove red blood cells prior to counting. Viability was determined by Trypan Blue exclusion.

i) Preparation of lymphocyte subpopulations.

An enriched T cell population was obtained from the spleen cells of treated or normal mice by panning on anti-mouse immunoglobulin plates according to the technique described by Mage et al., (1977) and modified by Wysocki and Sato (1978). Briefly, $30x10^6$ nucleated spleen cells were suspended in 3 ml phosphate buffered saline (PBS) containing 5% FCS. They were then poured onto sterile 15x100 mm polystyrene Petri dishes (Fisher Scientific Co., Don Mills, Ont.) precoated with 350 µg affinity-purified rabbit anti-mouse Ig in 10 ml 0.05M Tris (pH 9.5). Cells were allowed to adhere for 30 minutes at 4°C, were swirled, and allowed to incubate for another 30 minutes. An enriched T cell population was obtained by pooling the non-adherent cells collected by decanting the supernatant, and the cells obtained from two washings with PBS-1% FCS. The purity of the cell population was determined by mitogen assays, and was judged to be 90-95% T cell pure and 95% viable.

An enriched B cell population was obtained by depletion of T cells with monoclonal anti-Thy 1.2 antibody (Becton-Dickinson Co., Mountainview, CA) and Low Tox rabbit

complement (Cedarlane, Hornby, Ont.). Spleen cells were suspended in BSS at a concentration of $20x10^6$/ml and were incubated with an appropriate dilution of antibody (4 μg/ml) for 45 minutes on ice. The cells were washed once in BSS and resuspended in BSS containing complement at a final dilution of 1:10. Cells were incubated at 37°C in a water bath for 30 minutes, washed, resuspended in BSS, and recounted. This treatment killed 35-40% of the spleen cell population.

ii) Mitogen stimulation of spleen cell populations.

Enriched T and B cell subpopulations were tested for purity by stimulation with mitogen (Wysocki and Sato, 1978). Cells were suspended at a concentration of $5x10^6$/ml in RPMI 1640 medium (GIBCO) containing 10% FCS, 2mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml fungizone (complete medium). $5x10^5$ cells were grown for 30 h at 37°C in a 250 μl volume in the presence of either 2.5 μg E. coli 0128:B12 lipopolysaccharide (LPS)(Difco) or 0.25 μg concanavalin A (Con A) (Sigma). At the end of this period 1 μCi of ^{3}H-thymidine (New England Nuclear, Boston, MA) was added to each well for an additional 18 hours.

Cells were harvested onto glass fibre filters using a Skatron semi-automatic cell harvester (Mandel Scientific Co., Toronto, Ontario), and radioactivity was counted in a Beckman model LS 3801 liquid scintillation counter.

iii) In-vitro production of IFN-gamma.

IFN-gamma production was analyzed by growing $4x10^6$ cells/ml in 25 cm^2 flasks (Nunc-GIBCO) in 7 ml RPMI-1640 complete medium containing $5x10^{-5}$M 2-Mercaptoethanol (2-ME). Cultures grown in the presence of antigen received 50 µg/ml TNP-KLH or 10^9/ml DNP-BP. Thirty-six hours later, culture supernatants were collected by centrifugation, were filter-sterilized, and stored at -20°C until analyzed.

2.5 X-irradiation.

When required for certain experiments, recipient mice were exposed to 660 R from a Gammacell-20 irradiator (Atomic Energy of Canada Ltd., Ottawa, Ontario).

2.6 Adoptive transfer.

Single cell suspensions were transferred from normal or primed donor mice into normal or x-irradiated syngeneic recipients i.v. In co-adoptive transfer experiments, recipient mice received a mixture of cells from more than one donor. A more detailed description of cell transfers is presented in Results. Mice were challenged with the appropriate antigen in alum 2-3 hours after cell transfer.

2.7 Immunization and Bleedings.

Groups of 4-10 mice received i.p. injections of 10^9 DNP-BP (50 µg dry weight equivalent) alone or mixed with 2.5 mg alum ($Al(OH)_3$) in 0.5 ml volume. Mice injected with

other hapten-carrier conjugates received either 50 μg antigen in saline, or 2 μg DNP-OA or OA in alum. Further details are given in Results. Booster injections were given via the same route 4 weeks after the first injection.

Alum was prepared from $AlK(SO_4)_2$ and NaOH according to the method of Chase (1967). The stock preparation contained 10 mg dry weight/ml. In some instances, antigen was administered emulsified in CFA.

Murine anti-idiotypic antibodies were induced by immunization with 100 μg affinity-purified anti-DNP antibodies polymerized with glutaraldehyde (Eastman-Kodak). Briefly, 1 mg of an antibody preparation was polymerized by the addition of 35 μl of a 1:200 final dilution of glutaraldehyde in PBS pH 7. Visible polymerization (turbidity) occurred within 3-5 hours when the container was left covered at 20°C. The polymerization reaction was stopped by the addition of 1M lysine to reach a final concentration of 0.1M. Mice received an initial injection of 100 μg of antibody in CFA, followed by biweekly injections of 50 μg antibody in saline. Mice were bled 7 days after immunization.

In general, mice were bled under anesthesia by cardiac puncture at various intervals. The blood from all animals in a group was pooled, and the sera were stored at -20°C until needed.

Anti-TNP-KLH sera were prepared by injecting rabbits three times at 14 day intervals, with 5 mg TNP-KLH

emulsified in CFA. The injections were administered intradermally at multiple sites in the nuchal area. The animals were bled 10 days after the third injection and their sera were collected and stored at -20°C.

Rabbit anti-mouse IgE (RAME) was prepared by repeated immunization with affinity-purified mouse anti-DNP IgE mAb D12. A crude Ig preparation obtained by 35% ammonium sulfate precipitation (Campbell et al., 1970) was absorbed repeatedly over columns of Sepharose 4B coupled with normal mouse Ig and NMS, followed by affinity purification. This was carried out by absorption to and elution from a Sepharose 4B column coupled with a different mouse IgE mAb (TIB-142). The elution was with 0.1M DNP-Glycine pH 8.0. The antibody thus prepared showed no crossreactivity with other immunoglobulin isotypes in an antigen-specific EIA.

Rabbit anti-mouse IgG was prepared as previously described (Campbell et al., 1970). Briefly, pooled serum fractions were precipitated by ammonium sulphate and dissolved in borate-NaCl prior to dialysis against 0.0175M sodium phosphate buffer pH 7.5. Protein was estimated by optical density readings at 280 nm. This IgG enriched fraction was then separated on a column of Carboxymethyl Cellulose (CM 52)(Whatman Ltd., Maidstone Kent, England). Purity was determined by immunoelectrophoresis against normal mouse serum.

2.8 Immunosorbents.

The matrices used for antigen coupling included CNBr-activated Sepharose 4B (Seph) and CH-Sepharose (CH-Seph) (Pharmacia). Coupling with antigen was carried out according to the manufacturers' instructions. In brief, CNBr-Seph or CH-Seph were coupled with antigen at a ratio of 15 mg/g of matrix. The coupling was carried out in 0.1M $NaHCO_3$-0.5M NaCl pH 8.0 by rotation for 18 h at 4°C with 0.01% NaN_3.

In the case of Reacti-Gel(6X)(Pierce Chemical Co., Rockford, IL), 6 ml of washed and packed matrix were coupled with 30 mg DNP-BSA in 5 ml borate-NaCl buffer pH 9.2. The mixture was rotated for 30 h at 4°C after which the immunosorbent was treated as described above.

The silica affinity adsorbent (Boehringer Mannheim Canada, Dorval, Quebec) was coupled by rotating 0.5 g of matrix with 10 mg DNP-BSA in 0.15M NaCl, for 18 h at 4°C. After coupling, the immunosorbent was washed with 0.15M NaCl and blocked with 0.3M monoethanolamine pH 7.5. Subsequent washes were performed according to manufacturers' specifications. The DNP-BSA-silica was stored in 0.15M NaCl with 0.01% NaN_3 at 4°C.

2.9 Stabilization of immunosorbents with Glutaraldehyde.

In an attempt to stabilize the immunosorbent matrices used in this study, and reduce the amount of antigen leakage, a few batches of CNBr-Seph and Reacti-Gel (6X) were

stabilized with glutaraldehyde according to the method described by Kowal and Parsons (1980). Briefly, freshly coupled immunosorbents were equilibrated with 0.25M $NaHCO_3$ pH 8.8 prior to treatment with glutaraldehyde at a final concentration of 0.25% in 0.25M $NaHCO_3$ pH 8.8, for 1 h at room temperature, with rotation. The matrices were then sedimented by centrifugation (500xg) and washed with 4 volumes of 1M TRIS-HCl pH 7.8. They were then sequentially washed with 4 volumes of 1M NaCl-0.1% Tween 20 (Sigma) and 0.01M KPO_4-0.15M NaCl-0.001M ethylenediamine sodium tetraacetate (EDTA)-0.1% Tween 20 buffer pH 7.0. Immunosorbents were stored in the latter buffer at 4°C until tested.

2.10 Glutaraldehyde-polymerized immunosorbents.

Insoluble antigen immunosorbent was prepared by polymerizing 20 mg DNP-BSA in 2 ml sodium acetate-acetic acid buffer pH 5.0 by the dropwise addition of glutaraldehyde at a final concentration of 1% (Avrameas and Ternynck, 1969). The mixture was stirred for 10 min and allowed to stand for 50 min at room temperature. The reaction was stopped by the addition of 0.01M PBS pH 7.4. The polymerized antigen was disrupted by homogenization, washed by centrifugation in 0.1M Na_2CO_3, 0.2M glycine-HCl-0.5M NaCl pH 2.5, and finally in 0.2M sodium borate-0.5M NaCl pH 8.0. The polymer (DNP-BSA-POL) was stored at 4°C in borate-saline.

2.11 Affinity chromatography.

Normal serum, immune serum, buffer, or ascites, were rotated end-over-end with the various immunosorbents at a ratio of 3 ml serum or buffer, or 1 ml ascites, per 15 mg insolubilized antigen, for 2 h at room temperature. The absorbed fractions (effluent) were separated from the matrix by centrifugation, were dialyzed to PBS, and were stored at -20°C until needed. The extent of antibody depletion was determined by radioimmunoassay (RIA). Affinity-purified antibodies were obtained from the matrices by elution with 0.2M glycine-HCl-0.5M NaCl pH 2.5, or with 0.1M DNP-glycine in 0.2M Tris-HCl buffer pH 8.6. To prevent the denaturation of protein by the low pH elution, eluates were immediately neutralized by collection into tubes containing solid Tris. All eluates were extensively dialyzed against several changes of PBS at 4°C. In the case of hapten-eluted antibodies, a further dialysis against PBS pH 5.7 was performed. The eluates were then passed over a 10 ml column of Dowex 1-X8 anion exchange resin (20-50 mesh)(Biorad, Richmond, CA) to displace any residual hapten. After collection of the protein peak, all preparations were dialyzed to PBS pH 7.2. Affinity-purified antibodies were stored in small aliquots at -20°C until needed.

2.12 Reduction and alkylation.

MOPC-315 and MOPC-460 ascites were reduced and alkylated prior to affinity purification. Ascites were

dialyzed to 0.2M Tris-HCl pH 8.6 overnight at 4°C prior to reduction with 0.01M dithiothreitol (Eastman-Kodak) in Tris-HCl pH 8.6 for one hour at room temperature. The reaction mixture was neutralized by the addition of Tris-HCl pH 7.3 until the final pH reached 8.0. Thereafter, 0.011M Iodoacetamide (Aldrich Chemical Co., Milwaukee, WI) which was recrystallized from chloroform was added for 15 min at room temperature. The preparations were then dialyzed against borate-NaCl overnight at 4°C. Samples were filter-sterilized and stored at -20°C until used.

2.13 Gel Filtration.

Sephacryl S-300 superfine (Pharmacia) was equilibrated in 0.02M Tris HCl-0.5M NaCl pH 7.5 and loaded onto a 1.6x100 cm column (gel bed 1.6x80 cm). The sample was applied in a volume of 3 ml and was fractionated against gravity at a flow rate of 17 ml/h. Fractions were pooled as indicated in the results section, and were concentrated to the original volume (3 ml) by pressure ultrafiltration (Amicon, Danvers, MA) prior to dialysis against PBS.

2.14 Western blots.

Proteins were separated on sodium docedyl sulphate polyacrylamide gels (SDS-PAGE) prior to transfer onto nitrocellulose. SDS-PAGE was performed according to the method of Laemmli (1970), utilizing a 3% stacking gel and a 10% separating gel. Separation was achieved by running the

gels with cooling, at 30 mA per plate until the tracking dye reached the bottom of the plate. For reducing gels, samples were boiled for 1 min with 2-ME prior to separation.

Western blots were carried out essentially as described in the ProtoBlot Technical Manual (Promega Biotech, Madison, WI). Briefly, proteins were electrophoretically transferred from polyacrylamide gels to nitro-cellulose membranes (Schleicher and Schuell Inc., Keene, NH) using the Biorad transblot system, at 0.18 mA, for 16 hours at 4°C. The membrane was then washed and blocked with Tween-20 instead of BSA. Blots were stained with the appropriate antibodies as described in Results, and developed with the alkaline phosphatase-streptavidin BCIP/NBT substrate system (KPL, Gaithersburg, MD.).

2.15 Antibody determinations.

i) Passive Cutaneous Anaphylaxis.

Murine IgE antibodies were measured by rat passive cutaneous anaphylaxis (PCA), with a 48 hour latent period between the intradermal injection of 0.1 ml volumes of serially diluted serum and antigen challenge (Colby and Strejan, 1980). Rats were challenged i.v. with 2 mg DNP-BSA (for anti-DNP antibody determinations), or OA, in 1% Evans Blue dye. Titers were recorded as reciprocals of the highest dilution of serum giving a blueing reaction of 5 mm diameter or greater, 15-20 minutes after challenge. Four-fold differences in PCA titers between serum samples were

considered statistically significant.

ii) Immunoassays.

Anti-DNP and anti-OA IgG antibodies were determined using a solid phase radioimmunoassay (RIA) or an enzyme immunoassay (EIA). Anti-DNP and anti-OA IgG subclass determinations were made utilizing an EIA. Anti-idiotypic antibodies were detected utilizing an inhibition RIA.

ii a) Radioimmunoassay.

For the RIA, wells of a U-bottom polyvinyl microtiter plate (Dynatech Laboratories, Alexandria, VA) were coated with 50 μg/ml DNP-BSA in PBS for 18 h at 4°C. The plates were washed and blocked with PBS containing 1% BSA-0.2% Tween 20-0.02% sodium azide (RIA buffer). Serial dilutions of serum or affinity-purified antibodies in RIA buffer were added to the wells in duplicate or triplicate, and the plates were incubated for 2 h at room temperature. After washing, the plates were incubated with affinity-purified rabbit anti-mouse IgG for an additional 2 h at room temperature. Finally, the wells were incubated for 2 h with affinity-purified $Na^{125}I$-labelled goat anti-rabbit IgG (Miles). The wells were then washed, dried, cut and counted for radioactivity. The amount of antibody was calculated by extrapolation from a standard curve constructed with a mouse anti-DNP serum pool containing 2.5 mg antibody/ml.

RIA inhibition for anti-idiotypic antibody determination was performed as described by Geha (1982). In brief, a titration was first carried out to establish the

amount of anti-DNP antibody "idiotype" required to bind 70% of 20 ng of ^{125}I-DNP-BSA. This was carried out by coating wells of a microtiter plate with 100 µl volumes of serially diluted, affinity-purified mouse anti-DNP antibody, in PBS. The plates were incubated for 3 h at 20°C, then blocked and washed three times with RIA buffer. Finally, 20 ng ^{125}I-DNP-BSA was added to the wells at 20°C for the last 3 hours. At the end of the incubation period, the unbound antigen was removed, the wells were washed with RIA buffer, dried, cut and counted for radioactivity. The amount of "idiotype" required for 70% binding of 20 ng ^{125}I-DNP-BSA was between 1.25 and 2.5 µg/well. The inhibition of antigen-binding to Id was carried out by coating wells with 2.5 µg affinity-purified anti-DNP antibody. After discarding the unbound protein and blocking the wells with RIA buffer, 100 µl of serial dilutions of the sera or buffers passed through various immunosorbents were added to the wells, and the plates were incubated for 3 h at 20°C. After discarding the contents and washing with RIA buffer, 100 µl volumes of 20 ng ^{125}I-DNP-BSA were added for 3 h at 20°C. The wells were washed, dried, and counted as described above. The percent inhibition was calculated according to the following expression:

$$\frac{\text{cpm experimental} - \text{cpm background}}{\text{cpm total bound} - \text{cpm background}} \times 100$$

where cpm experimental are counts/min in the presence of inhibitor and cpm total bound are counts/min in the presence of RIA buffer.

ii b) Enzyme immunoassays.

The EIA procedure was performed essentially as described by Voller et al. (1980). For antigen-specific IgG determinations, flat-bottomed polystyrene microtiter wells (Dynatech) were coated with 100 µl of 100 µg/ml DNP-BSA or OA in PBS for 18 hours at 4°C. The wells were blocked with PBS containing 1% gelatin, for 1 hour at 20°C, and were washed with EIA buffer (0.1% gelatin-0.2% Tween 20-0.02% sodium azide). Thereafter, 100 µl volumes of serially diluted serum in EIA buffer were added to the wells and incubated at 20°C for 5-6 hours. The appropriate dilution of affinity-purified biotin-labelled goat anti-mouse IgG (Fc specific) (Jackson Immunoresearch - Bio/Can Scientific, Mississauga, Ontario) was then added to the wells for overnight incubation at 4°C. The wells were then incubated with alkaline phosphatase-conjugated streptavidin (SA-AP)(Jackson) for 1 hour at 37°C, and developed with p-nitrophenyl phosphate (Sigma 104 phosphate substrate tablets).

Sera analyzed for IgG subclass distribution were incubated with affinity-purified goat anti-mouse IgG1, or IgG2a (Southern Biotechnology Associates, Birmingham, AL). To amplify the read-out system, wells were washed and incubated for 2-3 hours at 20°C with biotinylated mouse anti-goat IgG (Jackson) followed by a 2 h incubation with SA-AP at 37°C. Plates were read in a Titertek Multiscan plate reader (Flow Laboratories, Mississauga, Ontario) at

405 nm. Total anti-DNP IgG was expressed in μg/ml following extrapolation from a curve constructed with a mouse anti-DNP serum standard. Due to the unavailability of antigen-specific subclass standards, anti-DNP IgG1 and IgG2a antibodies were expresseed in arbitrary units per well. One unit was defined as equal to an $O.D._{405}$ nm reading of twice background. Therefore, "x" units equal the dilution of the serum giving an $O.D._{405}$ reading of twice background, multiplied by that reading. Background readings ranged between 0.08 and 0.15.

For the antigen-leakage study, polystyrene U-bottom microtiter wells (Dynatech) were coated with either antigen or affinity-purified antibodies in PBS. The amounts used for coating are given in the "Results" section. Wells were washed and blocked, and serial dilutions of either mouse anti-DNP or rabbit anti-TNP serum in EIA buffer was then added to the wells. The plates were then incubated for 4-5 h at 20°C. After washing, an appropriate dilution of affinity-purified alkaline phosphatase-labelled goat anti-mouse IgG (Fc specific) (GAMG) or goat anti-rabbit IgG (Fc specific) (GARG) (Jackson) was added to the mouse or rabbit IgG respectively. The plates were then incubated for 5 h at 20°C or for 18 h at 4°C, washed and developed as described.

Detection of anti-idiotypic antibodies in EIA was accomplished by coating wells with affinity-purified anti-DNP monoclonal antibodies MOPC-315 (α), MOPC-460 (α), or a number of IgE hybridomas. Coated and blocked wells were

subsequently incubated with serial dilutions of non-absorbed or absorbed (idiotype depleted) serum, overnight at 4°C. After washing, the wells received an appropriate dilution of alkaline phosphatase-labelled goat anti-mouse IgG or IgM for 4 hours at 20°C. Wells were developed as described above. A more detailed description is presented in Results.

Antigen-specific IgE levels in hybridoma supernatants were determined by EIA. Supernatants were "enriched" for IgE by precipitation with 38% ammonium sulfate (Haba and Nisonoff, 1985), and concentrated by precipitation with 70% ammonium sulfate. The precipitates were redissolved in a small volume of PBS, and were extensively dialyzed against PBS prior to analysis by EIA. After incubation with the supernatants, antigen coated wells were incubated with an appropriate concentration of RAME (1:100 - 1:1000) for 12-16 hours at 4°C. This was followed by development with GARG-biotin (Jackson)(1:5000) for 12 hours at 4°C, and SA-AP (Jackson)(1:1000) for 2 hours at 37°C. The reaction was then visualized by the addition of substrate.

2.16 Radioiodination.

Ovalbumin, DNP-BSA, and goat anti-rabbit IgG were labelled with $Na^{125}I$ (Amersham Canada, Oakville, Ontario) by the chloramine-T method according to Yagi et al. (1963). In general, 1 mg protein was labelled with 0.5 mCi ^{125}I. Unbound iodine was removed using Sephadex G-25 gel filtration followed by dialysis against PBS.

2.17 Protein determinations.

The amount of protein in various preparations was determined according to Lowry et al. (1951) using a normal human serum as standard.

2.18 Passive transfer of serum.

Mice received 0.5 ml of serum i.v. 12 hours before and 12 hours after immunization with 2 μg DNP-OA in alum. Protein concentrations were adjusted such that each group received an equivalent amount (30-40 mg/ml).

2.19 Assays for interferon-gamma.

Interferon-gamma (IFN-gamma) was assayed in serum and spleen cell culture supernatants obtained from mice pretreated with DNP-BP, TNP-KLH, or from normal mice.

i) Viral plaque reduction assay.

Initial experiments utilized Vesicular stomatitis virus Indiana strain (VSV) and a monolayer of L-2 mouse fibroblast cells to detect IFN-gamma in the serum of mice (Colowick and Kaplan, 1981). Wells of a 6-well plate were coated with $5x10^5$ L-2 cells per well in Eagle's MEM (EMEM)(Flow Laboratories, McLean, VA) supplemented with 8% FCS for 14 hours at 37°C. After removal of the non-adherent L-2 cell overlay, dilutions of putative IFN-gamma-containing fractions were incubated for 4 hours at 37°C in 1 ml volume. At the end of this time, the supernatant was removed by aspiration, and 200 μl of VSV yielding 200-300 plaques/well

was added to the cell monolayer, with gentle shaking, for one hour at 37°C. Virus was removed by aspiration, and wells were coated with 2 ml of overlay medium (0.5% methyl-cellulose - EMEM) for 24 hours at 37°C, to allow plaque development. After discarding the overlay, cells and virus were fixed with 10% formaldehyde and stained with 1% Crystal Violet. Viral plaques were scored visually. The presence of IFN-gamma was confirmed by the sensitivity of the serum control IFN-gamma preparations to low pH dialysis (Nakamura et al., 1984).

ii) In-vitro bioassay for the detection of IFN-gamma.

The in vitro production of IFN-gamma was assayed by measuring growth inhibition of the murine B-cell lymphoma WEHI-279 (Reynolds et al., 1987). Briefly, WEHI-279 cells were maintained by regular passages in RPMI complete medium. For the assay, 10^4 cells from a culture in logarithmic growth phase were seeded in 0.1 ml volumes, to wells of a 96-well, flat bottomed tissue culture plate (Nunc-GIBCO), followed by the addition of serial dilutions of a recombinant-IFN-gamma standard (DNAX, Palo Alto, CA), or spleen cell culture supernatants, in 0.1 ml volumes. The cells were incubated for 72 hours at 37°C. 0.5 μCi ^{3}H-thymidine was added to each well for the last 18 hours of incubation. The presence of interferon-gamma in the cell-free supernatants was ascertained by the addition of 200 ng/ml anti-IFN-gamma mAb XMG1.2 (DNAX, Palo Alto, CA). In preliminary titrations it was determined that this was in

excess of the amount required to completely neutralize 10 U/ml of rIFN-gamma.

iii) Demonstration of IFN-gamma in-vivo.

To examine the effects of IFN-gamma on the in vivo anti-DNP IgE and IgG responses, groups of 4 mice were immunized with 10^9 DNP-BP i.p., followed 36 hours later by the i.v. injection of 1 mg XMG6 (monoclonal anti-IFN-gamma), J4.1 (monoclonal anti-NP), or saline. The mice were boosted 12 days later with 2 μg DNP-OA in alum. Additional details are given in Results. Mice were bled at 7 day intervals, and anti-DNP IgE and IgG subclass distribution was determined by PCA and EIA respectively.

2.20 Statistical Analysis.

PCA titers and IgG antibody levels were logarithmically transformed and analyzed by student's t test for differences between 2 means or by single factor analysis of variance (Zar, 1974). Differences between groups giving "p" values smaller than 0.05 were statistically significant. Otherwise, 4 fold differences or greater in PCA titers were considered statistically significant (Newburger et al., 1974). Sample calculations are presented in Appendix 1.

3. Results

3.1 Immunogenicity of DNP-BP.

To determine whether DNP-BP could stimulate anti-hapten IgE responses, groups of 4 CBA mice were injected i.p. with 10^9 DNP-BP (equivalent to 50 μg dry weight) either alone or adsorbed onto 2.5 mg alum. The mice were boosted 28 days later using the same immunization protocol. Figure 1 shows that DNP-BP failed to stimulate detectable anti-DNP IgE antibodies either alone or when injected with alum. Low PCA titers (1:10 - 1:40) were detected in sera obtained 7 days after booster (day 35) only in the group immunized with DNP-BP in alum. These decreased to undetectable levels by day 14 after booster (day 42). In contrast, IgG antibodies were elevated in both groups. For comparison, the PCA and IgG levels of sera from mice immunized with 2 μg DNP-OA in alum (the conventional protocol for IgE antibody induction) are also shown. No IgE antibodies to BP were detected, and IgG anti-BP antibodies were not determined.

The inability of DNP-BP to stimulate an anti-DNP IgE response could not be attributed to lack of immunogenicity of the conjugate since an excellent IgG response was detected. Moreover, as seen in Figure 2, the adoptive transfer of spleen cells from DNP-BP-primed donors together with cells from OA-primed donors to x-irradiated recipients, resulted in a typical secondary IgE response (PCA titer 1:1600) 7 days after booster with 2 μg DNP-OA in alum. In contrast, the transfer of spleen cells from OA-primed donors

Figure 1. Induction of IgE and IgG anti-DNP antibodies after immunization with DNP-BP. Mice were injected i.p. on days 0 and 28 (arrows) with 10^9 (50 μg) DNP-BP in saline (▲), 10^9 DNP-BP in alum (■) or 2 μg DNP-OA in alum (●).
A. IgE. B.IgG.

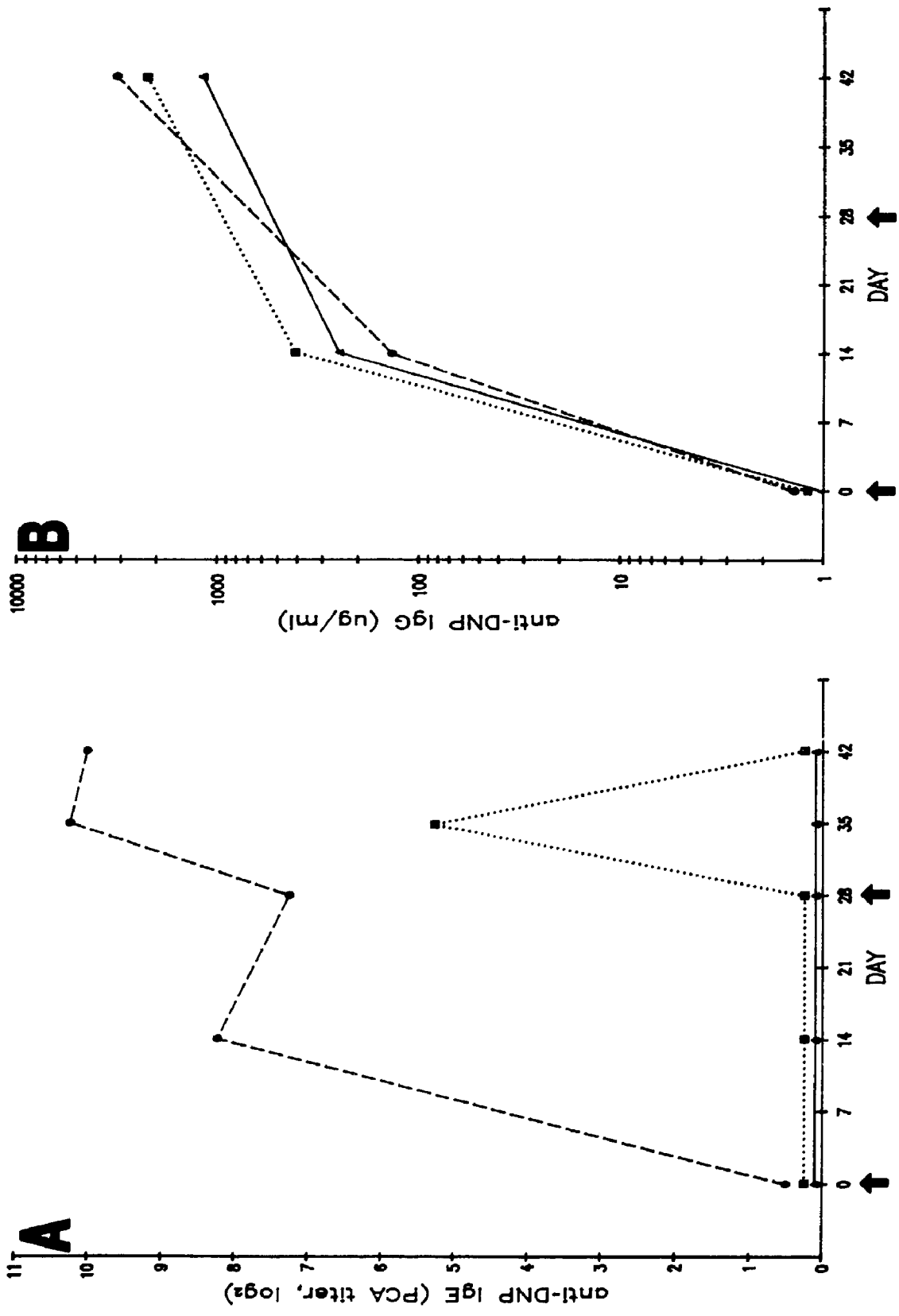
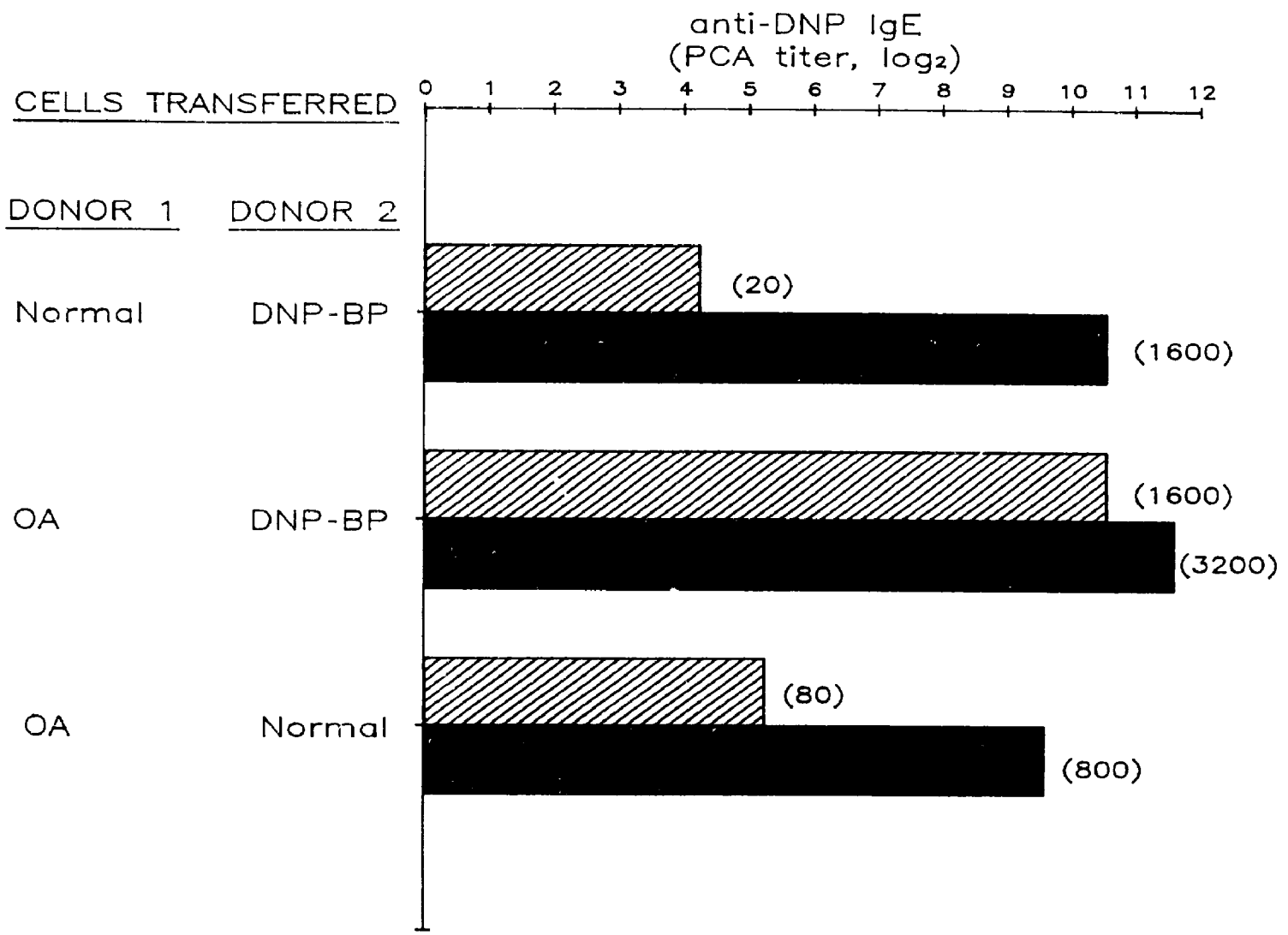

A
anti-DNP IgE (PCA titer, log2)
11
10
9
8
7
6
5
4
3
2
1
0
0
7
14
21
28
35
42
DAY
B
anti-DNP IgG (ug/ml)
10000
1000
100
10
1
0
7
14
21
28
35
42
DAY

Figure 2. Adoptive transfer of anti-DNP IgE. Donor-1 mice were primed with 0.5 μg OA in alum 7 days before transfer, or were left unprimed. Donor-2 mice were primed with 10^9 DNP-BP, 28 days before transfer, or were left unprimed. On the day of transfer, $2x10^7$ spleen cells from each donor were mixed and transferred i.v. to sublethally irradiated recipients which were boosted on the same day with 2 μg DNP-OA in alum i.p. PCA titers were determined on day 7 (hatched bar) and day 10 (solid bar) after booster (shown in brackets).

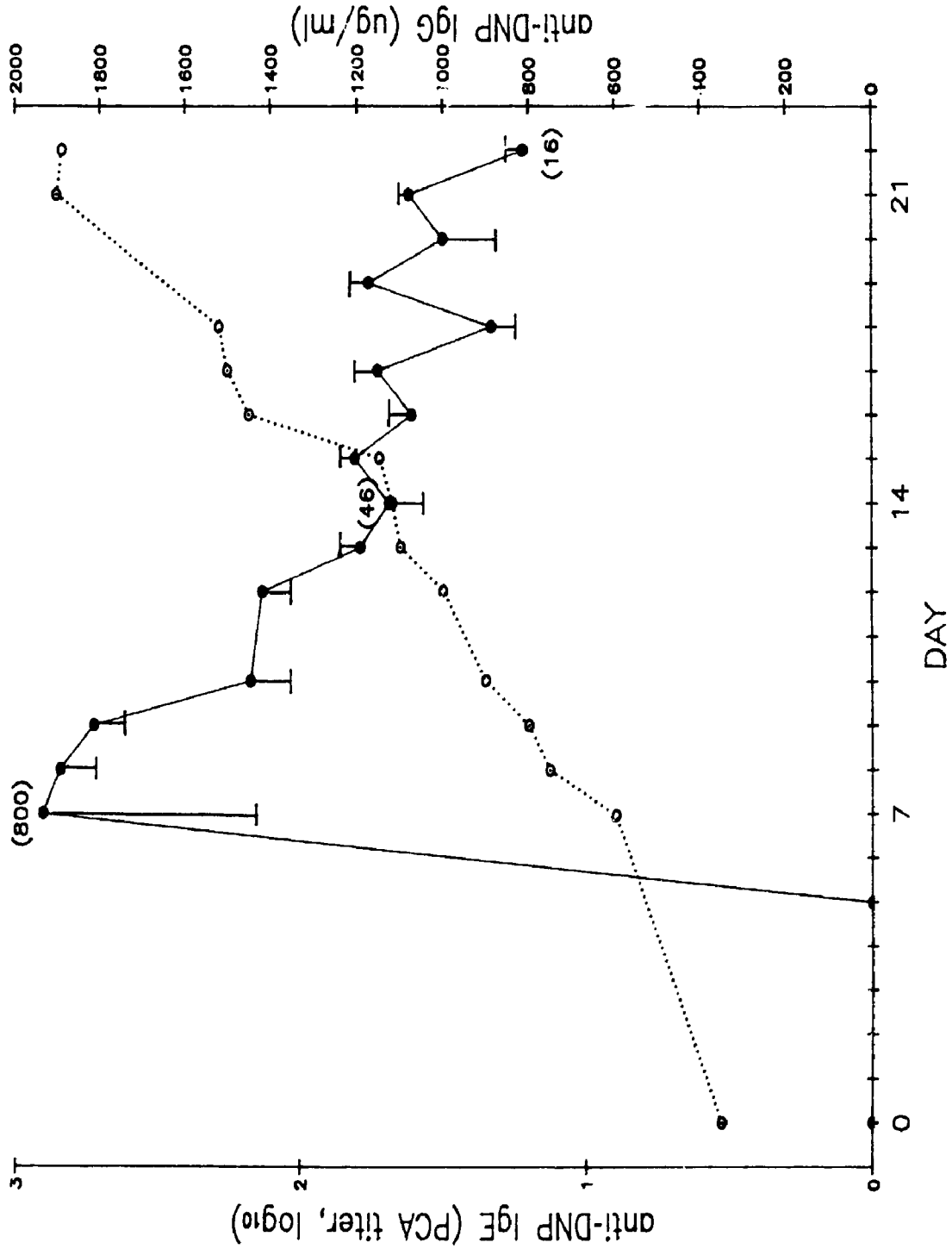

anti-DNP IgE
(PCA titer, log2)
0 1 2 3 4 5 6 7 8 9 10 11 12
CELLS TRANSFERRED
DONOR 1
DONOR 2
Normal
DNP-BP
(20)
(1600)
OA
DNP-BP
(1600)
(3200)
OA
Normal
(80)
(800)

together with normal spl[illegible] cells resulted in a day 7 anti-DNP-PCA titer of 1:80, typical for an accelerated primary response in the presence of carrier-primed helper T cells. These results clearly show that spleen cells from mice primed with DNP-BP contain DNP-specific IgE B memory cells, and suggested that the failure to respond to immunization with DNP-BP may have been due to an active mechanism of IgE-selective suppression.

3.2 Pretreatment with DNP-BP modulates anti-DNP IgE induced by immunization with DNP-OA.

One possible reason for the failure of the CBA mice to respond to immunization with DNP-BP could have been due to the induction of an active mechanism of IgE-selective suppression. In order to test this assumption, mice were pretreated with 10^9 DNP-BP two weeks prior to immunization with 2 μg DNP-OA in alum. The kinetics of 4 groups of 5 CBA mice bled on successive days after such treatment is presented in Figure 3. Anti-DNP IgE antibodies could not be detected prior to day 6 post DNP-OA immunization (not shown). However, mice produced elevated levels of anti-DNP IgE by day 7, after which time PCA titers decreased sharply, such that low titers (1:20-1:60) were always observed by days 13-14. By day 21, titers ranged between 1:10 and 1:20. In contrast to the kinetics of IgE, anti-DNP IgG levels continued to increase over the same period of time, reaching maximal concentrations by days 18-21. These results again

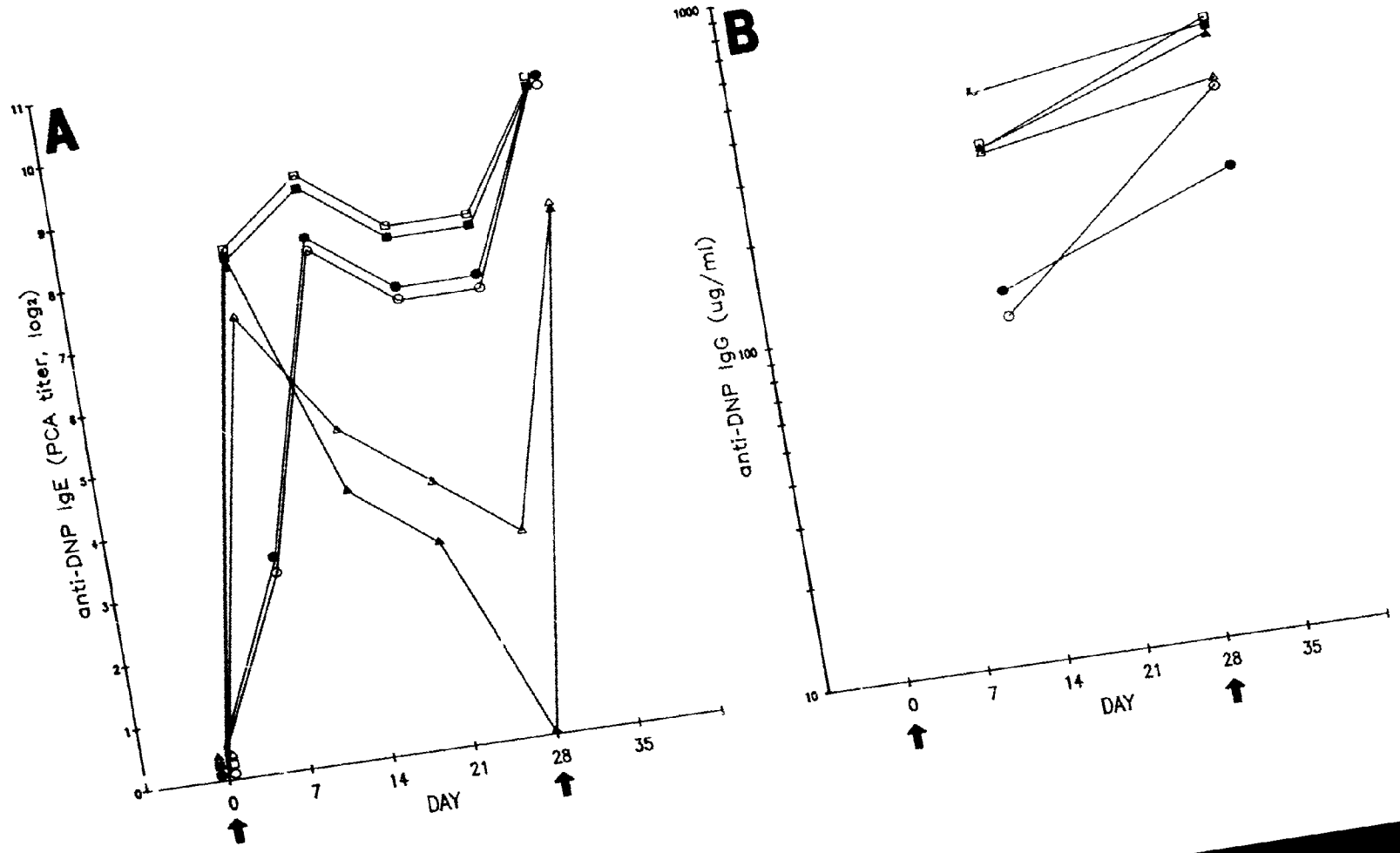
Figure 3. Effect of DNP-BP pretreatment on the anti-DNP IgE and IgG antibody response induced as a result of priming with DNP-OA. 4 groups of 5 CBA/J mice were pretreated with 10^9 DNP-BP on days -16, -14, and -12. On day 0, all mice were primed with 2 μg DNP-OA in alum. Groups of mice were bled on a rotating daily basis beginning on day 5, such that each group was bled every fourth day. Anti-DNP IgE values are $\log_{10}$ transformed PCA titers (●), and represent geometric means ÷/X S.E. Numbers in brackets represent PCA titers. Anti-DNP IgG concentrations were determined by radioimmunoassay on the serum pool of each sampling (O).


anti-DNP IgE (PCA titer, log10)
anti-DNP IgG (ug/ml)
DAY
(800)
(46)
(16)
0
7
14
21
1
2
3
200
400
600
800
1000
1200
1400
1600
1800
2000

demonstrate that DNP-BP was not deficient as an immunogen, since the anti-DNP IgE response induced as a result of immunization with DNP-OA appeared in an accelerated fashion (PCA titers of 1:320-1:800 by day 7). The sharp decline in anti-DNP IgE titers observed after day 7 is consistent with the development of a suppressor mechanism beginning to operate after the induction of IgE-B memory cells. Similar results were obtained when Balb/c mice were used (not shown).

The transient expression of serum anti-DNP IgE levels was a characteristic of pretreatment with DNP-BP, since mice pretreated with TNP-KLH or other DNP-carrier conjugates developed high day 7 anti-DNP IgE titers that did not decrease (Figure 4). Groups of 4 to 6 mice were pretreated with 10^9 DNP-BP once (day -14), or three times (days -16, -14 and -12), and were immunized with 2 μg DNP-OA in alum on day 0. Control groups were injected 3 times with 50 μg TNP-KLH, 50 μg DNP-Asc, 10^9 unconjugated BP, or saline, and were subsequently immunized with 2 μg DNP-OA in alum on day 0. The groups of mice pretreated with DNP-carrier conjugates exhibited an accelerated primary anti-DNP IgE response (day 7 PCA titer of 1:200 - 1:400), while the BP- or saline-pretreated groups developed a typical primary IgE response, (day 14 PCA titers of 1:400). By this time, in contrast to all other groups, mice pretreated with DNP-BP exhibited sharply decreased anti-DNP IgE levels which continued to decline until day 28. This characteristic day 7 IgE spike

Figure 4. Suppression of the anti-DNP IgE antibody response after treatment with DNP-BP. Groups of CBA/J mice were pretreated by i.p. injection with 10^9 DNP-BP on days -16, -14, -12 (▲), with 10^9 DNP-BP on day -14 (△), with 50 μg TNP-KLH on days -16, -14, -12 (■), with 50 μg DNP-Ascaris on days -16, -14, -12 (□), with 10^9 BP on days -16, -14, -12 (●) or with saline (○). All mice were primed on day 0 and boosted on day 28 with 2 μg DNP-OA in alum (arrcws). The results are from a typical experiment. A. IgE. B. IgG.


A
anti-DNP IgE (PCA titer, log2)
DAY
0
7
14
21
28
35
B
anti-DNP IgG (ug/ml)
1000
100
10
DAY

followed by suppression was a highly consistent and reproducible observation in numerous experiments (Appendix 1). A booster with 2 μg DNP-OA in alum on day 28 resulted in a secondary IgE response, but the anti-DNP PCA titers were 4-10 times lower than in any of the controls. The fact that anti-DNP IgG antibodies were not appreciably affected by DNP-BP pretreatment indicated that the suppressive mechanism was isotype selective.

The phenomenon described above was also highly dependent upon the physical coupling of the hapten to the bacterium, as pretreatment of mice with 10^9 BP mixed with 50 μg TNP-KLH did not induce the spike and suppression characteristic of pretreatment with DNP-BP (Table 1). Mice pretreated with saline or 10^9 BP prior to immunization with 2 μg DNP-OA in alum developed normal primary anti-DNP IgE responses, whereas mice primed with TNP-KLH, or BP and TNP-KLH, developed an accelerated primary response against the hapten (day 7 PCA titer 1:160 - 320). The suppressive mechanism was induced only in mice pretreated with 10^9 DNP-BP.

3.3 DNP-BP does not affect an established anti-DNP IgE response.

In addition to the above described effects, it was observed that DNP-BP was incapable of modulating an ongoing anti-DNP IgE response induced as a result of priming with DNP-OA in alum (Table 2). It can be seen that mice treated

Table 1.

Induction of anti-DNP IgE suppression requires linked recognition of the hapten-carrier conjugate.

Pretreatment[a]	anti-DNP IgE (PCA titer) day 0	7	14	21
Saline	nd[b]	<20	320	160
10^9 BP	<10	<20	640	320
10^9 DNP-BP	<10	160	20	10
50 μg TNP-KLH	nd	320	160	80
10^9 BP + 50 μg TNP-KLH	nd	160	80	80

a) Groups of 5 CBA/J mice were injected intraperitoneally with saline, 10^9 BP, 10^9 DNP-BP, 50 μg TNP-KLH or 10^9 BP mixed with 50 μg TNP-KLH on day -14. They were subsequently challenged with 2 μg DNP-OA in alum on day 0.

b) Not determined.

Table 2.

DNP-BP does not affect an established anti-DNP IgE response induced by priming with DNP-OA in alum.

Treatment on day 14[a]	anti-DNP IgE (PCA titer) day 14	21	28	35
DNP-BP	nd[b]	80	80	640
TNP-KLH	nd	160	160	1280
Saline	320	160	160	1280

a) Groups of 5 CBA/J mice were pretreated with 2 μg DNP-OA in alum on day 0. On day 14 they received intraperitoneal injections of either 10^9 DNP-BP, 50 μg TNP-KLH, or saline. All groups were boosted with 2 μg DNP-OA in alum on day 28 after bleeding.

b) Not determined.

with TNP-KLH or DNP-BP 14 days after priming with DNP-OA in alum did not have significantly different PCA titers than mice treated with saline. Even after a booster immunization with 2 μg DNP-OA in alum on day 28, the day 35 PCA titers were not significantly different (1:640 vs 1:1280).

3.4 The environment of the DNP-BP pretreated mouse is necessary for the observed IgE spike and suppression.

Sublethally irradiated recipients of spleen cells from DNP-BP treated but not DNP-OA-primed donors developed unsuppressed anti-DNP IgE levels when challenged with DNP-OA in alum on the day of transfer (not shown). However, when cells from mice primed with DNP-OA in alum were transferred into groups of DNP-BP- or TNP-KLH-pretreated recipients, the transferred cells came under the regulatory mechanisms of the host. It can be seen that by day 14 after immunization with DNP-OA in alum, anti-DNP IgE levels were significantly reduced in the DNP-BP-pretreated host (Table 3). The cells transferred into the TNP-KLH-pretreated recipients however, were not significantly affected.

3.5 The suppression induced by DNP-BP is DNP-specific.

The results presented in Table 4 showed that the suppression induced as a result of DNP-BP pretreatment was hapten-specific, since the pretreatment of mice with DNP-BP did not affect the development of anti-OA IgE levels when mice were immunized with 2 μg DNP-OA or 2 μg OA in alum.

Table 3.

Regulation of anti-DNP IgE production by the environment of DNP-BP pretreated mice.

Pretreatment[a]	anti-DNP IgE (PCA titer) day 7	day 14
DNP-BP	320	40
TNP-KLH	320	160
saline	160	320
control[b]	640	40

a) Recipient mice were pretreated with 10^9 DNP-BP, 50 μg TNP-KLH, or saline 14 days prior to transfer. On the day of transfer, they received 50×10^6 spleen cells from donor mice primed with 2 μg DNP-OA in alum 28 days prior to transfer. Recipients were primed with 2 μg DNP-OA in alum 2 hours after cell transfer.

b) Control mice were pretreated with 10^9 DNP-BP, and were boosted with 2 μg DNP-OA in alum. They did not receive DNP-OA primed cells.

Table 4.

Effect of DNP-BP pretreatment on anti-OA IgE antibodies.

Pretreatment[a]	Priming[b]	PCA titer	
		day 7	day 14
Saline	OA	40	320
TNP-KLH	OA	40	320
DNP-BP	OA	40	320
Saline	DNP-OA	ND[c]	160
TNP-KLH	DNP-OA	ND	160
DNP-BP	DNP-OA	ND	160

a) Groups of 4 CBA/J mice were injected on days -16, -14 and -12 with saline, 50 µg TNP-KLH or 10^9 (50 µg) DNP-BP i.p.

b) Mice were injected on day 0 either with 2 µg OA or with 2 µg DNP-OA, both adsorbed onto 2.5 mg $Al(OH)_3$.

c) Not determined.

Further evidence for the specificity of the suppressive mechanism was obtained from groups of mice pretreated with NIP-BP. Mice immunized with 2 μg NIP-DNP-OA in alum produced anti-DNP IgE titers that were not significantly different from those of mice pretreated with saline (Table 5). The group of mice pretreated with NIP-BP displayed elevated day 7 anti-DNP IgE levels as compared to the saline pretreated group, and although the day 14 PCA titers were lower, by day 21 there was no difference. It is interesting to note that the anti-DNP IgE response in mice immunized with NIP-DNP-OA in alum was not as high as in mice immunized with DNP-OA in alum. This is possibly due to antigenic masking of the DNP-groups by subsequent labelling with NIP. The reason for the differences in the day 21 anti-DNP PCA titers between the saline and NIP-BP-pretreated groups immunized with DNP-OA (1:160 vs 1:1280) is unclear, however, it does not detract from the fact that pretreatment with NIP-BP did not reduce anti-DNP IgE levels.

3.6 The suppressive mechanism is not the result of the induction of T suppressor cells.

In order to determine whether the failure to sustain elevated levels of anti-DNP IgE antibodies after pretreatment with DNP-BP and priming with DNP-OA was due to the activity of suppressor cells, spleen cells were transferred from donor mice already having gone through the characteristic day 7 spike (Table 6). Normal recipients

Table 5.

Pretreatment of mice with NIP-BP does not suppress anti-DNP IgE induced as a result of immunization with NIP-DNP-OA.

		anti-DNP IgE (PCA titer)		
Pretreatment[a]	Challenge[b]	day 7	14	21
Saline	DNP-OA	20	320	160
NIP-BP	DNP-OA	20	640	1280
Saline	NIP-DNP-OA	<10	80	40
NIP-BP	NIP-DNP-OA	40	20	40

a) Groups of 5 CBA/J mice were injected with saline or with 10^9 NIP-BP on days -16, -14, -12.

b) Mice were immunized with 2 μg DNP-OA or 2 μg NIP-DNP-OA in alum i.p. on day 0.

Table 6.

The anti-DNP IgE suppression induced by DNP-BP pretreatment is not mediated by adoptive transfer of spleen cells.

Cells transferred[a)]	anti-DNP IgE (PCA titer) day 7	day 14
DNP-BP, DNP-OA	640	320
TNP-KLH, DNP-OA	320	320
Normal	10	320

a) Normal recipients received 40×10^6 spleen cells from DNP-BP- or TNP-KLH-pretreated donors 10 days after priming with 2 μg DNP-OA in alum. The recipients were then boosted with 2 μg DNP-OA in alum, and were bled on days 7 and 14.

received $40x10^6$ spleen cells obtained from DNP-BP- or TNP-KLH-pretreated mice 10 days after priming with DNP-OA in alum, and were then challenged with 2 μg DNP-OA in alum. There were no differences in the day 14 PCA titers of these two groups (1:320). Thus, cells transferred from DNP-BP-pretreated donors were not capable of suppressing the IgE response of recipients. Moreover, the transferred cells were themselves restimulated by day 7 (PCA titer 1:640), as the group of mice receiving normal spleen cells did not respond to immunization by day 7 (PCA titer 1:10).

In a more elaborate experiment of a similar nature, it was observed that enriched T cell populations from mice pretreated with DNP-BP and then primed with DNP-OA in alum were not capable of inducing long term IgE suppression. Recipient mice were sublethally irradiated, and then received cells as outlined in Table 7. Enriched B cell populations were taken from donors 14 days after pretreatment with DNP-BP, whereas enriched T cell populations were obtained from pretreated mice 10 days after priming with DNP-OA in alum. It is evident that the groups receiving T cells from mice pretreated with DNP-BP had reduced anti-DNP IgE levels on day 7 relative to transfer (PCA titers 1:320 - 1:640), but this suppression was transient in nature in that it was not evident by day 14. The elevated day 7 anti-DNP IgE titers (1:2000) observed in mice receiving a mixture of normal B cells and DNP-OA in alum primed T cells, is probably the result of an

Table 7.

The anti-DNP IgE response is not regulated by T suppressor cells induced as a result of pretreatment with DNP-BP.

Cells transferred[a]

Pretreatment		Pretreatmer [b]	anti-DNP IgE (PCA titer)		
Donor 1[c]		Donor 2[d]	day 7	day 14	day 21
normal B	+	saline T	2000	4000	4000
DNP-BP B	+	DNP-BP T	640	4000	4000
DNP-BP B	+	TNP-KLH T	4000	4000	4000
TNP-KLH B	+	DNP-BP T	320	8000	8000
DNP-BP B	+	saline T	8000	8000	4000

a) Recipients were sublethally irradiated (6[illegible] Rad) prior to receiving a mixture of $12x10^6$ enriched T cells and $18x10^6$ [illegible] cells. They were then challenged with 2 μg DNP-OA in alum 2 hours after cell transfer, and bled on days 7, 14 and 21.

b) All donors of T cells were immunized (primed) with 2 μg DNP-OA in alum, 12 days after pretreatment.

c) Donor 1 mice were pretreated with 10^9 DNP-BP, 50 μg TNP-KLH, or saline 14 days prior to transfer. B cells were enriched by treatment of spleen cells with anti-Thy 1.2 and complement prior to transfer.

d) Donor 2 mice were pretreated as above. Spleen cells obtained 10 days after priming with 2 μg DNP-OA in alum were enriched for T cells by panning on anti-mouse Ig coated plates (see Materials and Methods).

accelerated primary response.

3.7 The passive transfer of absorbed serum from mice pretreated with DNP-BP does not suppress IgE levels.

In a previous study, it was suggested that the pretreatment of mice with DNP-BP induced a suppressive mechanism that could be transferred by anti-DNP-depleted serum (Essani, 1983; Essani et al., 1984). The absorption of serum from suppressed mice over DNP-immunosorbent was thought to release auto-anti-idiotypic antibodies from their corresponding serum idiotype. Those antibodies were invoked as being the cause for the observed suppression in that study. However, it was subsequently demonstrated that the passive transfer of DNP-absorbed sera of mice pretreated with DNP-BP and primed with DNP-OA in alum were incapable of suppressing recipient mice following challenge with 2 μg DNP-OA in alum (Table 8). In numerous repeat experiments, no differences were found in the PCA titers between groups of mice receiving absorbed NMS, or absorbed serum from suppressed mice (PCA titer 1:320).

The passive transfer of serum from mice suppressed as a result of DNP-BP priming could however prevent the development of an anti-DNP IgE response in recipient mice if transferred without absorption. This suppressive capacity was found to depend on the concentration of anti-DNP IgG in the sample (Table 8). It was determined that sera containing as little as 150 μg/ml anti-DNP IgG were capable

a) Groups of 5 CBA/J mice were treated with saline, normal mouse serum (NMS), or serum obtained from mice pretreated with DNP-BP and primed with DNP-OA in alum 21 days earlier. Treatment was administered intravenously 12 hours before and after recipient immunization with 2 μg DNP-OA in alum.

b) Samples were injected untreated or after absorption on antigen-coupled Sepharose (Seph.) columns.

c) Total concentration of anti-DNP IgG injected per recipient.

d) Anti-DNP IgE titers were determined on day 14 relative to immunization of recipients with 2 μg DNP-OA in alum.

e) Normal mouse serum.

f) Non-absorbed serum.

g) Absorbed serum.

h) Not determined.

Table 8.

Inhibition of the anti-DNP IgE antibody response by passive transfer of serum is the result of anti-DNP IgG antibody.

Preparation[a]	Treatment[b]	μg anti-DNP IgG[c]	day 14 PCA[d]
Saline	none	0	640
NMS[e]	none	0	320
NAS[f]	none	850	20
NAS	none	425	40
NAS	none	210	80
NAS	none	106	320
NAS	none	53	320
AS[g]	OA Seph.	800	40
AS	DNP-BSA Seph.	35	320
AS	DNP-BSA + OA Seph.	35	160
AS	DNP-BSA + KLH Seph.	40	320
AS	DNP-OA Seph.	nd[h]	320

of preventing the development of an anti-DNP IgE response when administered 12 hours before and 12 hours after immunization with 2 μg DNP-OA in alum. The observed suppression was however not unique to the serum of mice pretreated with DNP-BP, as any serum having this threshold concentration of DNP-specific IgG was suppressive (not shown).

As DNP-BP-pretreated mice primed with DNP-OA in alum always developed a day 7 anti-DNP IgE spike prior to suppression, and since the anti-DNP IgE response was apparently the only isotype suppressed, it was reasoned that the IgE spike might be instrumental in the suppression observed. Thus, conditions were created which simulated an anti-DNP IgE spike in pretreated mice prior to their immunization with DNP-OA in alum. Mice pretreated with DNP-BP, or untreated mice, received a total of 300 μg of an anti-DNP IgE mAb mix, or of an anti-NIP IgE mAb mix, 6,4, and 2 days prior to immunization with 2 μg DNP-OA in alum. As the results in Table 9 indicate, the passive transfer of IgE did not significantly alter the day 7 PCA titers of any group. Therefore, it was apparent that anti-DNP IgE, or IgE of an irrelevant antigenic specificity (NIP), did not contribute to the suppression of the IgE response induced as a result of pretreatment and priming. Furthermore, the results indicated that IgE did not feedback inhibit as was the case with the passive transfer of anti-DNP IgG.

Table 9.

Passive transfer of anti-DNP IgE does not inhibit the development of an anti-DNP IgE response.

Recipient treatment[a]		anti-DNP IgE (PCA titer)	
Pretreatment	IgE transferred	day 7	day 14
DNP-BP	anti-DNP	160	80
DNP-BP	anti-NIP	160	40
DNP-BP	nil	160	40
TNP-KLH	anti-DNP	160	80
TNP-KLH	anti-NIP	160	80
TNP-KLH	nil	320	160
Saline	anti-DNP	10	320
Saline	anti-NIP	20	160
Saline	nil	40	320

a) Groups of 5 CBA/J mice were pretreated with 10^9 DNP-BP, 50 μg TNP-KLH, or saline on day -14. They received a total of 300 μg anti-DNP IgE in 3 injections, 6, 4, and 2 days prior to immunization with 2 μg DNP-OA in alum. The anti-DNP IgE was a mixture of the ascitic fluid from two hybridomas having specifity for either NIP (hybridomas D2.1/E + F3.2/E) or for DNP (hybridomas 3/B9 + D12). The IgE in the ascitic fluid was enriched prior to passive transfer using precipitation with ammonium sulfate at 38% saturation.

The results in this study concur with those of Essani (1983) in terms of the immunogenicity of DNP-BP, the kinetic pattern of anti-DNP IgE expression after pretreatment and priming, and the inability to demonstrate the existence of suppressor T cells. However, the results of the present study differ from those of Essani (1983) in terms of the passive transfer of suppression via DNP-depleted serum, and the proposed mechanism of suppression.

3.8 Evidence for the existence of auto-anti-idiotypic antibody was the result of antigen leakage from DNP-BSA-Sepharose.

Based on the suppressive capacity of absorbed serum from DNP-BP-pretreated mice, and the observation that the suppressive activity eluted together with the bulk of the immunoglobulins when separated on Sephadex G-200, it was previously suggested that the suppression of the anti-DNP IgE response might occur as the result of the induction of auto-anti-idiotypic antibodies in the serum of the DNP-BP-pretreated mice (Essani, 1983; Essani, et al., 1983). In order to test this assumption, a solid-phase radioimmunoassay was developed. In that assay, the presence of anti-idiotypic antibodies was determined by their ability to inhibit the binding of ^{125}I-DNP-BSA to affinity-purified anti-DNP antibody-coated plates (idiotype). The anti-DNP antibodies were obtained by elution from immunosorbent matrices, of day 21 sera from mice pretreated with DNP-BP

and primed with DNP-OA in alum.

The results in Figure 5 demonstrate that the absorption of sera from mice pretreated with DNP-BP and primed with DNP-OA on a DNP-BSA CNBr-Sepharose column clearly inhibited the binding of radiolabelled antigen to the idiotype coated wells in a dose-dependent fashion. However, it was also apparent that absorbed NMS inhibited this interaction, whereas non-adsorbed sera did not. Table 10 shows that the passage of three different pools of normal mouse serum, of two pools of anti-DNP serum, or even of RIA buffer through DNP-BSA-Sepharose, resulted in significant inhibition of ^{125}I-DNP-BSA binding to anti-DNP antibody coats. The inhibition ranged between 35% and 59% for the undiluted effluents and was obvious even at a dilution of 1:16. In contrast, the normal or anti-DNP serum pools or buffers that were not passed through DNP-BSA-Sepharose had very little or no inhibitory activity. As a control, normal mouse serum was passed through OA-Sepharose, and this too was without inhibitory effect (not shown). The observed inhibition was independent of the amount of antibody used for coating. In order to determine whether the release of antigen from Sepharose-immunosorbents was unique to DNP-BSA, OA was coupled to CNBr-activated Sepharose.

Figure 6 shows that NMS rotated for 2 hours at 20°C with OA-Sepharose according to the manufacturers' recommendations, inhibited the binding of radiolabelled-OA to affinity-purified mouse anti-OA antibodies, in a fluid

Figure 5. Inhibition of ^{125}I-DNP-BSA binding to affinity-purified mouse anti-DNP antibody by sera passed through DNP-BSA-Sepharose. Wells coated with 2.5 μg affinity-purified polyclonal mouse anti-DNP antibodies were incubated for 3 h at room temperature with 100 μl volumes of serially diluted mouse anti-DNP serum pools passed through DNP-BSA-Sepharose (△), anti-DNP serum not passed (▲), normal mouse serum pool passed through DNP-BSA-Sepharose (□), or normal mouse serum not passed (■). Total cpm bound in the absence of inhibitor (●).

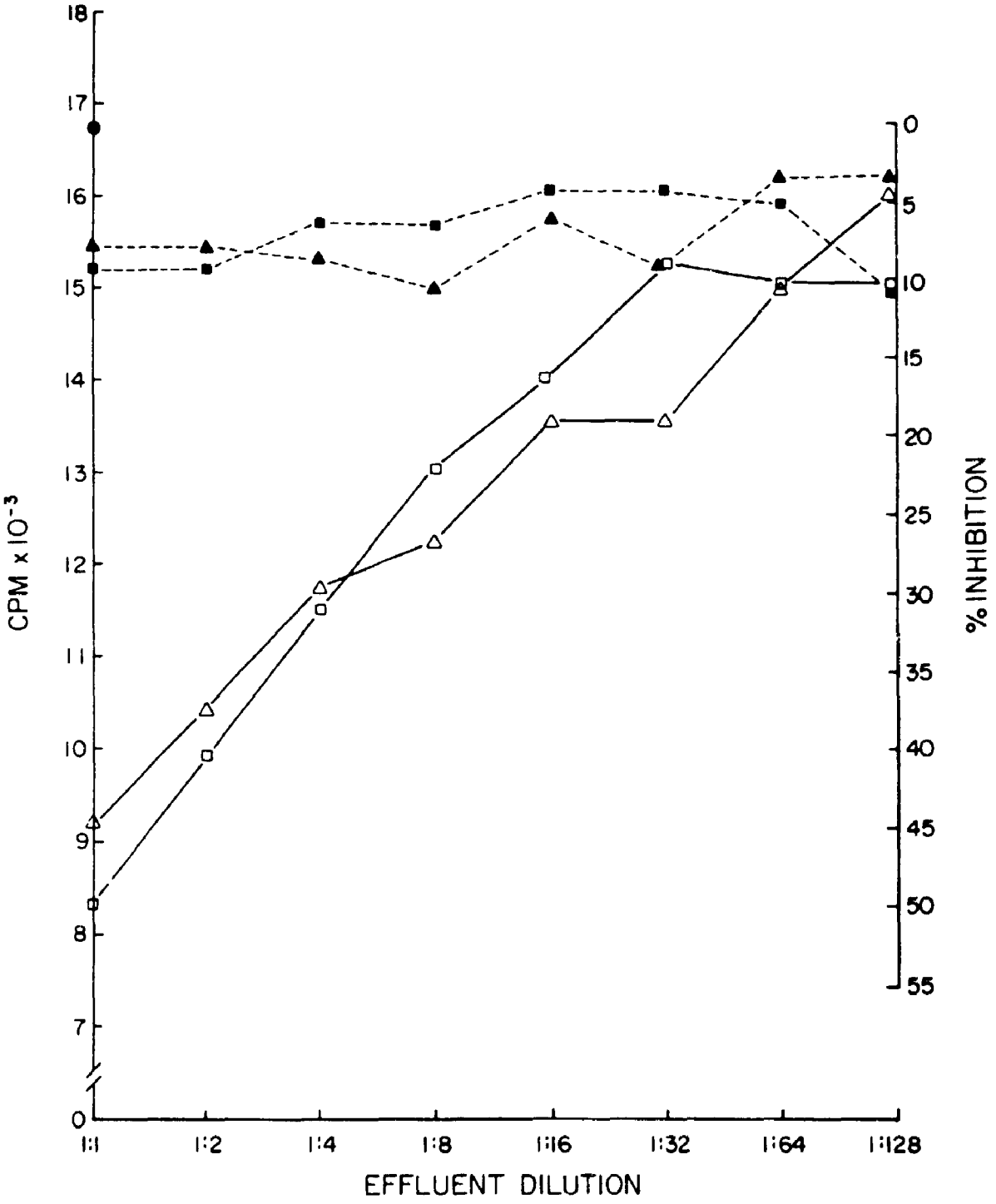

18
17
16
15
14
13
12
11
10
9
8
7
0
CPM x 10⁻³
0
5
10
15
20
25
30
35
40
45
50
55
% INHIBITION
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
EFFLUENT DILUTION

Table 10.

Inhibition of ^{125}I-DNP-BSA binding to anti-DNP antibodies by various DNP-BSA-Sepharose effluents.

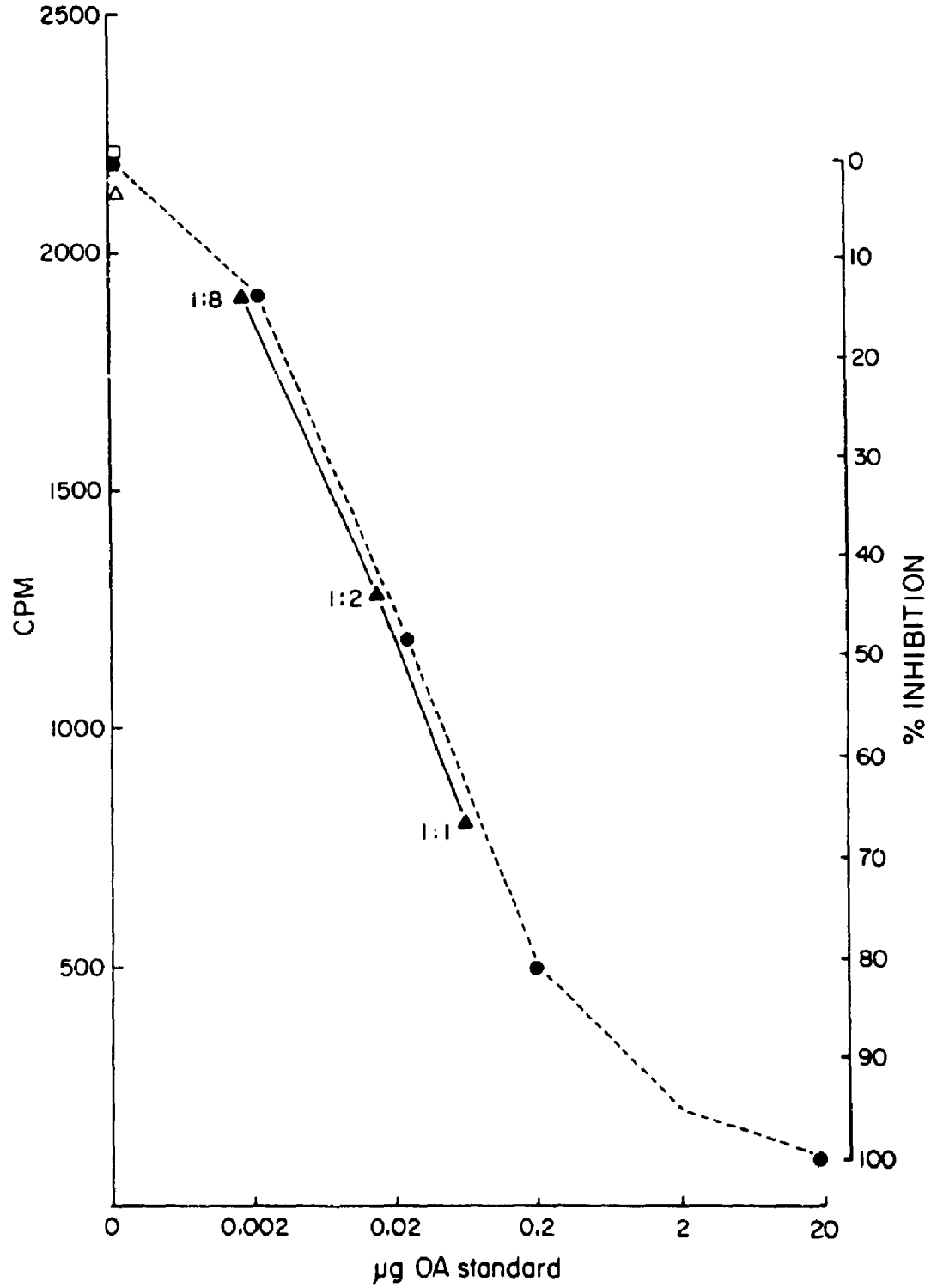
Inhibitor[a]	Dilution	Sepharose	cpm	% Inhibition
NMS-A[b]	1:1	None	27240	5
NMS-A	1:1	OA	26667	7
NMS-B	1:1	None	26434	8
NMS-C	1:1	None	24243	15
Anti-DNP pool[c]	1:1	None	25040	13
Buffer	1:1	None	28673	0
Anti-DNP pool	1:1	DNP-BSA	18586	35
Anti-DNP pool	1:4	DNP-BSA	21076	26
NMS-A	1:1	DNP-BSA	15770	45
NMS-B	1:1	DNP-BSA	12043	58
NMS-C	1:1	DNP-BSA	17204	40
Buffer	1:1	DNP-BSA	11756	59

a) Microtiter wells were coated with 2.5 µg affinity-purified anti-DNP antibodies and then incubated with 0.1 ml inhibitor for 3 h at room temperature. After washing, the wells were incubated with 20 ng ^{125}I-DNP-BSA for an additional 3 h at room temperature.

b) Three different pools (A,B and C) of normal mouse serum were tested.

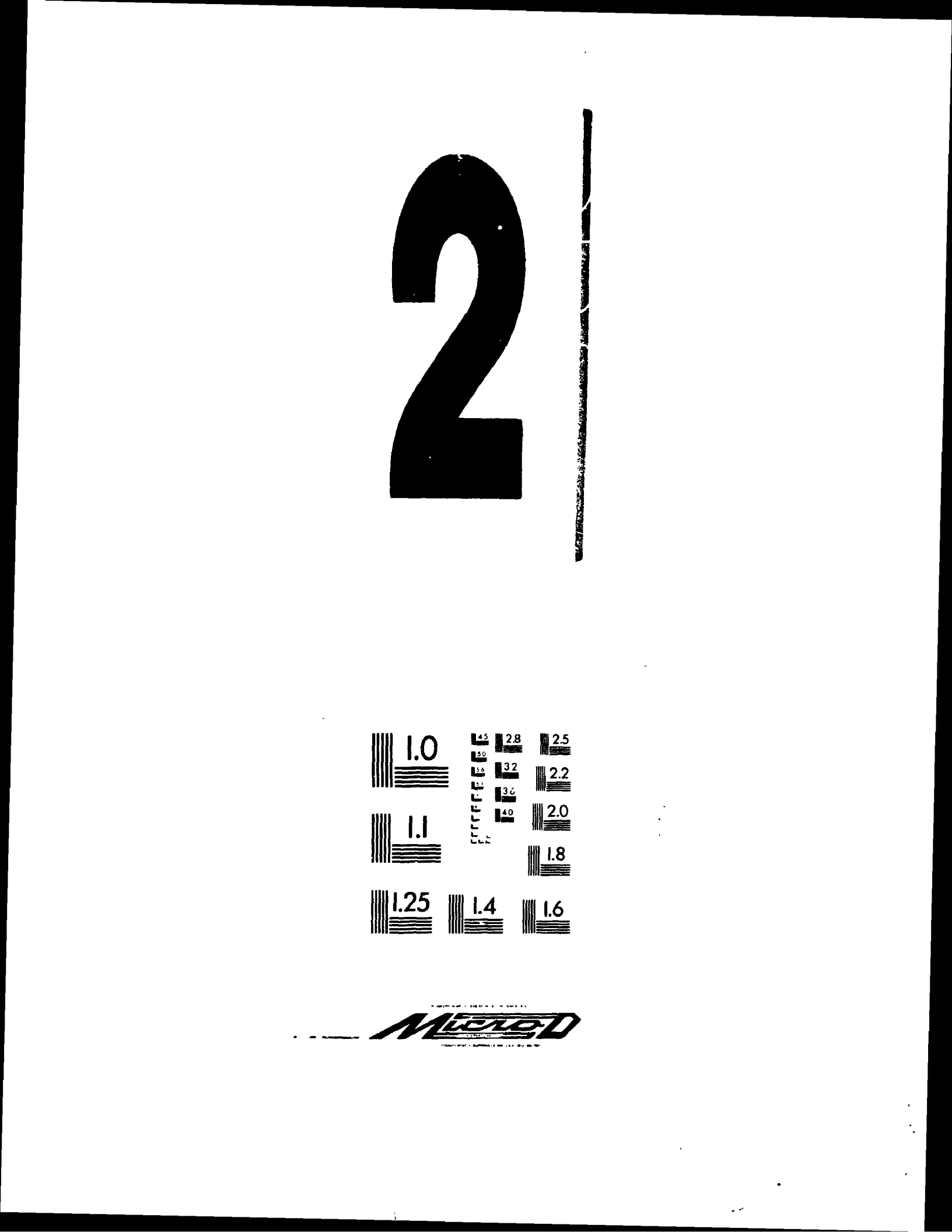
c) Serum pool from mice immunized with DNP-OA.

Figure 6. Inhibitory activity of normal mouse serum (NMS) effluent from OA-Sepharose immunosorbent. Inhibition of antiserum binding was carried out by a fluid-phase radio-immunoassay. A standard inhibition curve was constructed after incubating 0.045 μg ^{125}I-OA with 0.77 μg, affinity-purified mouse anti-OA antibodies in the presence of increasing amounts of unlabelled OA, for 1 h at 37°C and 18 h at 4°C, followed by precipitation with goat anti-mouse IgG serum (●). The extent of ^{125}I-OA binding was determined for effluents of NMS passed through OA-Sepharose (▲), unpassed NMS (△), and for buffer (□). The amount of antigen per ml in the OA-Sepharose effluent can be estimated by extending a vertical line from any dilution of effluent to the abscissa and multiplying by the dilution X10.


2500
2000
1500
1000
500
CPM
1:8
1:2
1:1
0
10
20
30
40
50
60
70
80
90
100
% INHIBITION
0
0.002
0.02
0.2
2
20
µg OA standard

phase radioimmunoassay, in a dose-dependent fashion. After extrapolation to the standard inhibition curve, the amount of OA released from the immunosorbent was calculated to be 0.36 μg/ml. The inhibitory activity of these Sepharose adsorbed effluents could not be decreased by centrifugation at 12,000xg for 30 minutes. Based on the notion that DNP-BSA released from the immunosorbent could be separated from immunoglobulin by virtue of their molecular weight differences, 3 ml of a normal mouse serum pool was rotated for 2 hours at 20°C with 3 ml DNP-BSA-Sepharose (15 mg DNP-BSA). The effluent was then dialyzed for 18 hours through a 12 kDa cut-off membrane, against 0.02 M Tris-HCl buffer pH 7.5, and applied to a 1.6x80 cm Sephacryl S-300 column. After elution, the fractions were pooled as shown in Figure 7, and each pool was concentrated to the original volume (3 ml), dialyzed against PBS, and tested by RIA inhibition. As is evident from the results obtained, significant amounts of inhibitory activity were detected over a wide range of molecular weight, indicating that effective separation between the antigen and the immunoglobulin in the effluent could not be achieved by gel filtration. Based on these results, it was apparent that the inhibition observed in the radioimmunoassays employed in earlier studies could be attributed to the presence of antigen in the absorbed serum samples.

Figure 7. Elution profile and inhibitory activity of DNP-BSA-Sepharose-passed mouse serum after Sephacryl S-300 gel filtration. Fractions were pooled as indicated (horizontal bars). Inhibition of ^{125}I-DNP-BSA binding to affinity-purified polyclonal anti-DNP antibody is shown by the solid vertical bars. Inhibitory activity of unfractionated serum is shown by the hatched bar.


2
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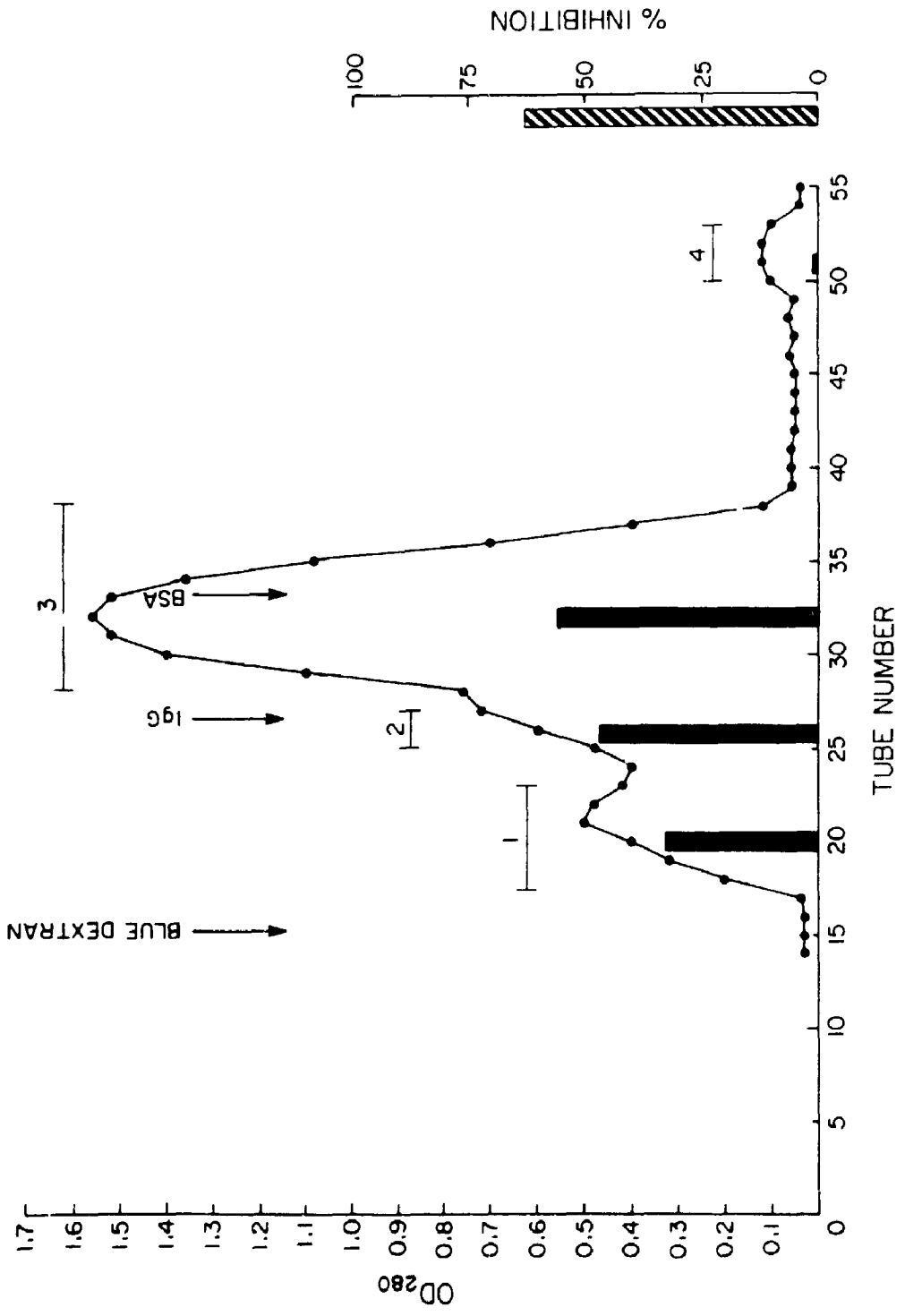
1.25
1.4
1.6
2.8
2.5
2.2
2.0
1.8
MICROCOPY


% INHIBITION
100
75
50
25
0
TUBE NUMBER
OD_{280}
BLUE DEXTRAN
IgG
BSA
1
2
3
4

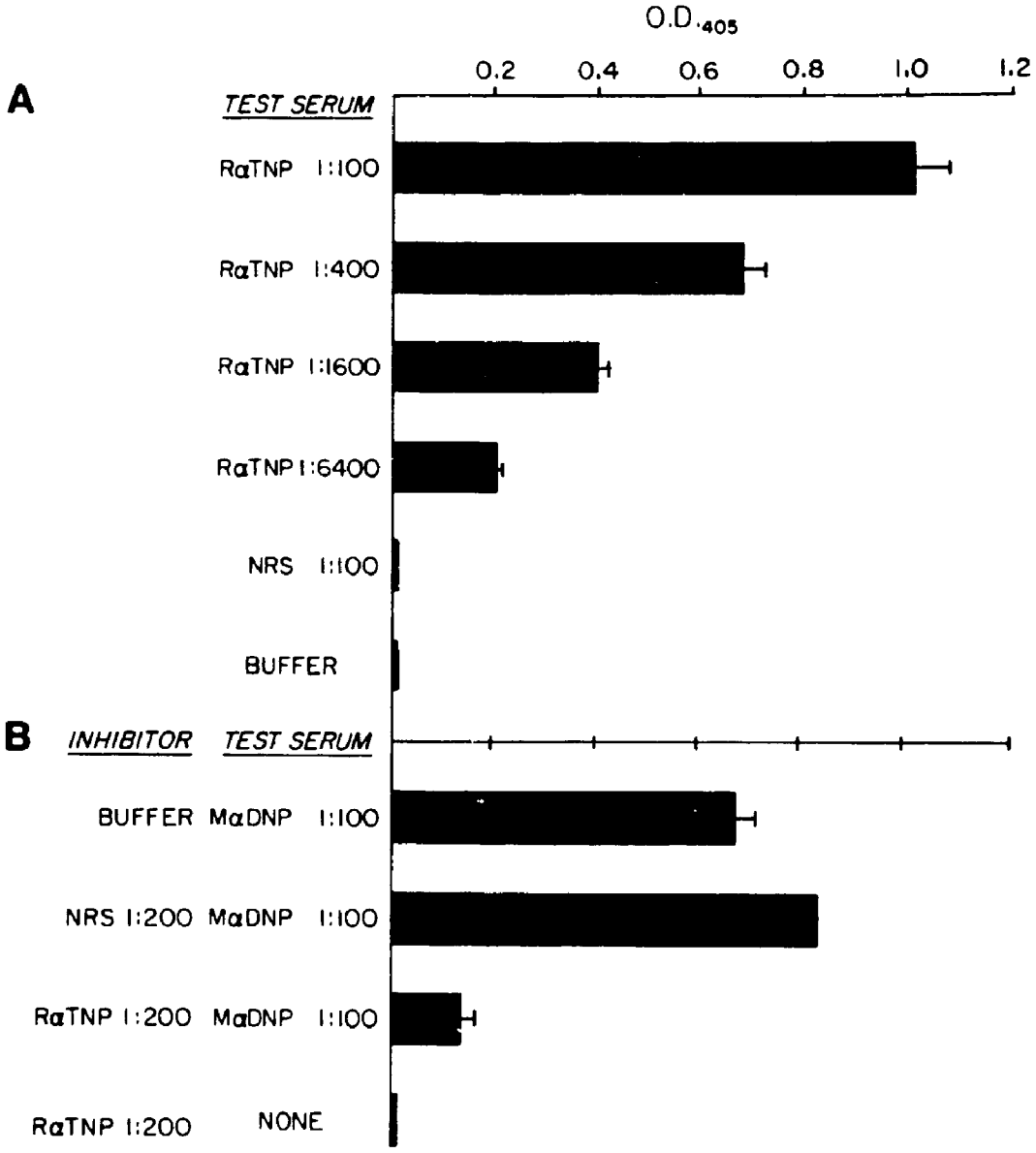
3.9 Demonstration of antigen in affinity-purified antibody preparations.

In order to determine whether or not affinity-purified antibodies contained immunosorbent-released antigen, wells of microtiter plates were coated with 10 µg/ml reduced and alkylated MOPC-315, an anti-DNP IgA myeloma, which was affinity-purified from ascites by elution with DNP-glycine from a DNP-BSA-Sepharose column. The wells were incubated with serial dilutions of rabbit anti-TNP serum and then were developed with alkaline phosphatase-GARG and substrate. The results in Figure 8A clearly show that rabbit anti-TNP serum bound to affinity-purified MOPC-315 in a dose-dependent fashion. Moreover, rabbit anti-TNP could inhibit the binding of mouse anti-DNP serum to the MOPC-315 coat by over 60%, indicating that both preparations bound to the same ligand (Figure 8B). The binding was obviously specific since neither normal rabbit serum nor buffer showed any activity.

3.10 Antigen release from various insoluble matrices.

The above results demonstrated that significant amounts of antigen were released from CNBr-Sepharose immunosorbents, and that these could be detected in both the effluent and eluate fractions of anti-DNP containing sera. In an attempt to resolve this problem and establish the radioimmunoassay inhibition assay as a reliable test for the detection of anti-idiotypic antibodies, a number of immunosorbent

Figure 8. Demonstration of antigen released from DNP-BSA Sepharose immunosorbents by enzyme immunoassay. A: Binding of rabbit anti-TNP antibodies. Wells were coated with 10 μg/ml affinity-purified MOPC-315 ascites. Elution was with 0.1 M DNP-glycine in 0.2 M Tris pH 8.6. The wells were blocked with 1% gelatin and incubated in triplicate with serial dilutions of rabbit anti-TNP-KLH serum. The second antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (Fc specific). B: Binding of mouse anti-DNP-antibodies. Wells were coated with a 1:2 dilution of PBS passed through DNP-BSA-Sepharose. The wells were incubated with either EIA buffer or rabbit anti-TNP-KLH diluted 1:200, for 2 h at room temperature (inhibitor). After washing, the wells were incubated with mouse anti-DNP-OA serum diluted 1:100 for 18 h at 4°C followed by alkaline phosphatase-conjugated goat anti-mouse IgG (Fc specific).


O.D.405
0.2
0.4
0.6
0.8
1.0
1.2
A
TEST SERUM
RαTNP 1:100
RαTNP 1:400
RαTNP 1:1600
RαTNP 1:6400
NRS 1:100
BUFFER
B
INHIBITOR
TEST SERUM
BUFFER MαDNP 1:100
NRS 1:200 MαDNP 1:100
RαTNP 1:200 MαDNP 1:100
RαTNP 1:200 NONE

matrices were tested for antigen leakage. The following matrices were prepared and compared to DNP-BSA-Sepharose: DNP-BSA-Sepharose stabilized by glutaraldehyde, Reacti-Gel (6X) with and without glutaraldehyde stabilization, DNP-BSA-affinity adsorbent (silica), glutaraldehyde-polymerized DNP-BSA, DNP-Lys-coupled CH-Sepharose, and DNP-Lys-Sepharose. PBS was absorbed with rotation on these matrices for 2 hours at 20°C. The immunosorbent-passed PBS was then coated onto microtiter wells, and antigen leakage was determined by development of the wells with either rabbit or mouse anti-DNP serum. In titration, it was determined that both these anti-sera could detect as little as 0.01 μg/ml DNP-BSA coat. Table 11 shows that the wells coated with PBS from DNP-BSA immunosorbents all had detectable levels of antigen. The stabilization of DNP-BSA-Sepharose with glutaraldehyde reduced only slightly the amount of antigen released, and there was very little difference between effluents passed through stabilized or unstabilized Reacti-Gel (6X). In contrast, very little antibody bound the PBS effluents obtained from DNP-Lys-substituted matrices.

A similar pattern was observed in the case of radioimmunoassay inhibition (Table 12). Effluents that did not inhibit the binding of ^{125}I-DNP-BSA to the anti-DNP antibody coat were those obtained from the DNP-Lys-coupled CH-Sepharose, DNP-Lys-Sepharose, and from DNP-BSA polymerized with glutaraldehyde.

Table 11.

Release of antigen from various immunosorbent matrices.

Immunosorbent[a]	O.D.$_{405}$[b] Mouse anti-DNP[c]	Rabbit anti-DNP[d]
None	0.00	0.00
Sepharose	2.0	2.0
Sepharose/GA[e]	1.32 + 0.03	0.53 + 0.04
R-6X[f]	1.66 + 0.19	1.7 + 0.3
R-6X/GA	1.24 + 0.1	1.8 + 0.16
DNP-Lys-CH-Sepharose[g]	0.35 + 0.01	0.10 + 0.01
DNP-Lys-Sepharose[h]	0.22	0.04

a) Microtiter wells were coated with 0.2 ml immunosorbent-passed PBS for 18 h at 4°C and were blocked with 1% gelatin. In each case, 3 ml PBS were rotated with 1 g DNP-BSA- or DNP-Lysine-coupled matrices.

b) Determined by enzyme immunoassay.

c) The coated wells were incubated with mouse anti-DNP-OA serum diluted 1:200 followed by alkaline phosphatase-conjugated goat anti-mouse IgG (Fc specific).

d) The coated wells were incubated with rabbit anti-TNP-KLH serum diluted 1:200, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Fc specific).

e) DNP-BSA-Sepharose stabilized with glutaraldehyde.

f) DNP-BSA-Reacti-Gel (6X).

g) DNP-Lysine-CH-Sepharose.

h) DNP-Lysine-CNBr-Sepharose.

Table 12.

Demonstration of antigen by inhibition in effluents passed through various immunosorbent matrices.

Immunosorbent[a]	Dilution	%Inhibition
None	1:1	0
Sepharose[b]	1:1	60
	1:40	20
Sepharose/GA[c]	1:1	35
	1:40	13
R-6X[d]	1:1	32.5
R-6X/GA[e]	1:1	16
Silica[f]	1:1	81
	1:40	11
DNP-BSA-POL[g]	1:1	4
DNP-Lys-CH-Sepharose[h]	1:1	3

a) Microtiter wells were incubated with 2.5 µg affinity-purified mouse anti-DNP antibody. The wells were blocked and incubated with 0.1 ml immunosorbent-passed RIA buffer for 3 h at room temperature, followed by washing and addition of 20 ng ^{125}I-DNP-BSA.

b) Unless otherwise stated, all matrices were coupled with DNP-BSA.

c) Stabilized with glutaraldehyde.

d) Reacti-Gel (6X).

e) Reacti-Gel (6X) stabilized with gluaraldehyde.

f) Affinity-adsorbent (Silica)

g) DNP-BSA polymerized by glutaraldehyde.

h) DNP-lysine coupled to CH-Sepharose.

3.11 Lack of radioimmunoassay inhibition by sera of mice pretreated with DNP-BP and immunized with DNP-OA in alum.

Immunosorbent matrices coupled with the monovalent hapten DNP-Lys, and protein antigens cross-linked by polymerization with glutaraldehyde were found to release negligible amount of reactive antigen when tested in radioimmunoassays or EIA. Both DNP-Lys-Sepharose and DNP-Lys-CH-Sepharose matrices did however release significant amounts of hapten as detected spectrophotometrically (not shown). This was readily removed by dialysis, and as seen in Table 12, did not inhibit the binding of ^{125}I-DNP-BSA to anti-DNP antibody. Based on these results, sera from mice pretreated with DNP-BP or TNP-KLH and primed with DNP-OA in alum were absorbed on these matrices. After absorption, the sera, and affinity-purified antibodies eluted with DNP-Glycine, were passed over a column of Dowex 1-X8 anion exchanger to remove any residual hapten, and were dialyzed extensively to PBS prior to testing in the radioimmunoassay inhibition test. The results of numerous absorptions with many different sera clearly demonstrated that sera absorbed under these conditions did not inhibit the binding of ^{125}I-DNP-BSA to anti-DNP antibody coats, regardless of the pretreatment (Table 13). As controls for the validity of the radioimmunoassay inhibition test as an assay for the detection of anti-idiotypic antibodies, wells were coated with the monoclonal antibodies MOPC-315 or MOPC-460, or the eluted anti-DNP antibodies from DNP-immunosorbents. The

a) A radioimmunoassay inhibition test was performed, in which the ability of absorbed serum fractions or anti-idiotypic antibodies to inhibit the binding of ^{125}I-DNP-BSA to various anti-DNP antibody coats was measured in counts per minute. Total CPM added was 48,900.

b) Putative anti-idiotypic antibody preparations were added to antibody coated wells (idiotype) at a dilution of 1:2, for a total of 4 hours at room temperature.

c) Wells were coated with 0.5 µg reduced and alkylated affinity-purified MOPC-315 in PBS.

d) Wells were coated with 0.5 µg reduced and alkylated affinity-purified MOPC-460 in PBS.

e) Wells were coated with 2.5 µg anti-DNP antibody eluted from DNP-Lys-CH-Sepharose columns. Antibodies were induced by pretreating mice with DNP-BP on days -16, -14, and -12, and priming with 2 g DNP-OA in alum on day 0. Sera were obtained on day 21.

f) Anti-DNP and anti-OA antibody-depleted serum from mice pretreated with DNP-BP, were obtained 21 days after DNP-OA priming.

g) Normal mouse serum absorbed with DNP-Lys-CH-Sepharose and OA-Sepharose.

h) Anti-MOPC-315 antibodies were obtained by immunization of mice with affinity-purified, reduced and alkylated MOPC-315.

i) Anti-MOPC-460 antibodies were obtained by immunization of mice with affinity-purified, reduced and alkylated MOPC-460.

j) Mice were immunized with glutaraldehyde-polymerized affinity-purified polyclonal mouse anti-DNP antibodies eluted from DNP-Lys-CH-Sepharose.

Table 13.

Lack of inhibition by the sera of mice pretreated with DNP-BP after absorption with DNP-Lys-CH-Sepharose.

	CPM[a]		
Serum added[b]	MOPC-315[c]	MOPC-460[d]	Serum eluate[e]
None	25,600	23,400	28,000
ADS 1[f]	24,900	nd	26,300
ADS 2	nd	nd	28,200
ADS 3	nd	nd	26,850
ADS 4	nd	nd	27,900
ADS 5	nd	nd	28,150
adsNMS[g]	25,400	22,900	27,600
anti-MOPC-315[h]	2,100	19,800	28,400
anti-MOPC-460[i]	21,200	6,300	28,100
anti-Serum eluate[j]	26,100	22,650	18,400

results demonstrate that the appropriate anti-idiotypic antibody could reduce the amount of radiolabelled antigen binding the coated wells. The least amount of inhibition was observed for the eluted polyclonal antibody coat and the induced anti-idiotypic antibody (44%).

3.12 Utilization of anti-DNP hybridomas to detect the presence of auto-anti-idiotypic antibodies in the serum of DNP-BP-pretreated mice.

As the failure to demonstrate auto-anti-idiotypic antibodies in the serum of pretreated mice may have been due to low concentrations of specific idiotype, or anti-idiotype, hybridomas were constructed for analysis. Several attempts to isolate an anti-idiotypic hybridoma from the spleens of suppressed mice were unsuccessful (as measured by radioimmunoassay inhibition). Therefore, in an attempt to isolate a predominant idiotype from IgE-suppressed mice, a number of anti-DNP IgG1 hybridomas were produced from several fusions. These were used in EIA's for the screening of auto-anti-idiotypic antibodies. In no instance, did absorbed sera bind anti-DNP IgG1 monoclonal-antibody coated wells (not shown). Similarly, negative results were obtained when the myeloma proteins MOPC-315 or MOPC-460 were used as idiotype coats.

The failure to detect anti-idiotypic antibodies may have been due to the absence of the appropriate idiotypic specificity from the hybridomas constructed. Since IgE was

the isotype suppressed as a result of pretreatment and priming, it was felt that the idiotypic determinants might be restricted to IgE. Therefore, 5 anti-DNP IgE-producing hybridomas were constructed. These were initially screened by PCA, and subsequently shown to be of the IgE isotype by EIA and Western blot analysis (Figure 9). Figure 9 also demonstrates the specificity of the rabbit anti-mouse IgE antibody (RAME) for the IgE isotype. The anti-DNP IgE monoclonals did not cross-react with anti-IgG, IgM, or IgA developing reagents in antigen-specific EIA (Table 14).

The results in Table 15 show that a number of absorbed sera were capable of binding IgE coated wells. Although it appeared that these sera bound preferentially to the 3/B9 idiotype, the total number of sera binding this monoclonal antibody was small (8%). Furthermore, sera binding this idiotype did so weakly, as evident by the low O.D. readings, and usually also bound more than one monoclonal antibody (Table 15). In no instance however, did non-absorbed or normal mouse serum bind the IgE coated wells. All attempts to demonstrate 3/B9 and D12 in the sera of pretreated and suppressed mice were unsuccessful. In both EIA (Table 16), and Western blot analysis (not shown), anti-3/B9 antiserum and anti-D12 mAb were not capable of detecting their corresponding idiotypes in the sera of mice pretreated with DNP-BP. Titration of 3/B9 with anti-3/B9 and D12 with anti-D12 indicated that these assays were sensitive in the range of 10 ng/ml. Based on the above observations, and on

Figure 9. Purity of anti-DNP IgE hybridoma 3/B9, and isotype specificity of RAME. 3/B9 (lanes A, C, E), and normal mouse IgG (lanes B, D, F), were separated on a 10% polyacrylamide gel prior to transfer to nitro-cellulose. Lanes A and B were stained with a 1:200 dilution of affinity-purified RAME for 6 hours at room temperature, and developed with alkaline phosphatase labelled Goat anti-rabbit IgG at 1:5000 overnight at 4°C. Lanes C and D were developed with alkaline phosphatase labelled goat anti-mouse IgG (Fc specific) at 1:5000 overnight at 4°C. Lanes E and F were stained with non-purified RAME at 1/20, followed by alkaline phosphatase labelled goat anti-rabbit IgG at 1:5000. Staining was visuallized using BCIP/NBT.


— epsilon
— gamma
— light
A
B
C
D
E
F

Table 14.

Isotype of anti-DNP hybridomas.

Hybridoma[b]		O.D.$_{405}$[a] anti-IgG[c]	anti-IgM[d]	anti-IgA[e]	RAME + GARG[f]
3/B9	-	0.032	0.063	0.082	0.917
1/C12	-	0.066	0.081	0.071	0.622
1/F8	-	0.033	0.072	0.080	0.709
1/D8	-	0.042	0.055	0.062	0.588
D12	-	0.062	0.031	0.029	0.408
TIB-142	-	0.021	0.022	0.029	0.988
AC6#93	-	1.112	0.101	0.101	0.069
MOPC-315	-	0.109	0.203	1.138	0.054

a) Wells were coated with 10 µg/well DNP-BSA for an antigen-specific EIA measuring the isotypic nature of hybridomas. The EIA was developed with alkaline phosphatase labelled reagents having specificity for the isotypes listed below.

b) Affinity-purified mAb from a number of different hybridomas was added at µg/well for 2 hours at RT.

c) Wells were developed with alkaline phosphatase labelled Goat anti-mouse IgG (Fc specific) added at 1:6000.

d) Wells were developed with alkaline phosphatase labelled Goat anti-mouse IgM (Fc specific) added at 1:5000.

e) Wells were developed with alkaline phosphatase labelled Goat anti-mouse IgA added at 1:5000.

f) Wells were developed with RAME at 2 µg/ml, followed by alkaline phosphatase labelled Goat anti-rabbit IgG at 1:4000.

a) EIA wells were coated with affinity-purified anti-DNP IgE hybridomas. The binding of serum to these coats was measured by development with goat anti-mouse IgG biotin, and SA-AP. Values given have been corrected for background, and are values above those obtained with NMS on the individual coats.

b) Serum added to antibody coated wells was obtained from mice pretreated with 10^9 DNP-BP on days -16, -14, and -12 relative to immunization with 2 μg DNP-OA in alum. Absorbed serum (ADS), or non-absorbed serum (NAS), from both Balb/c and CBA mice, were added at a 1:3 dilution for 4 hours at room temperature.

c) EIA wells were coated with 0.5 μg anti-DNP IgE monoclonal antibody in PBS overnight at 4°C.

d) CBA mice were immunized with anti-DNP antibody eluted from DNP-Lys-Sepharose (see Materials and Methods). The antiserum was added at a 1:1000 dilution.

e) Mouse (B/C) anti-D12 ascites partially purified by precipitation with 38% ammonium sulfate was added to the IgE coated wells at a dilution of 1:2,500.

Table 15.

Demonstration of binding of absorbed sera to anti-DNP IgE hybridomas.

	O.D.$_{405}$[a]				
Serum added[b]	3/B9[c]	D12	1/D8	1/C12	1/F2
NAS +14 (B/C)	.090	.027	<0.0	.006	<0.0
NAS +21 (B/C)	.016	.002	<0.0	.005	<0.0
ADS +14 (B/C)	<0.0	.000	<0.0	.007	<0.0
ADS +21 (B/C)	.116	.042	<0.0	<0.0	.011
NAS +14 (CBA)	.023	.005	.000	.012	.013
NAS +21 (CBA)	.008	.003	<0.0	.009	.002
ADS +14 (CBA)	.103	.084	.033	.048	.022
ADS +21 (CBA)	.516	.142	.043	.123	<0.0
anti-serum[d] (CBA)	.127	.075	.075	.083	.078
anti-D12[e] (B/C)	<0.0	.639	<0.0	<0.0	<0.0

Table 16.

Screening of idiotype by EIA.

Coat[a]		Serum[b]		anti-serum[c]		O.D.$_{405}$
3/B9	-	Nil	-	Nil	-	0.028
3/B9	-	Nil	-	anti-3/B9	-	0.917
3/B9	-	ADS	-	anti-3/B9	-	0.878
3/B9	-	NAS	-	anti-3/B9	-	0.983
3/B9	-	NMS	-	anti-3/B9	-	0.926
D12	-	Nil	-	Nil	-	0.034
D12	-	Nil	-	anti-D12	-	0.761
D12	-	NAS	-	anti-D12	-	0.691
D12	-	NMS	-	anti-D12	-	0.644

a) Wells were coated with 5 μg/well affinity-purified anti-DNP IgE mAb 3/B9 or D12 in PBS overnight at 4°C.

b) After blocking, wells received 100 μl of a 1:2 dilution of serum obtained from mice pretreated with DNP-BP and primed 7 days earlier with DNP-OA in alum (NAS), serum depleted of anti-DNP antibodies by immunoabsorbtion (ADS), or normal mouse serum (NMS).

c) Wells then received anti-3/B9 antiserum (1:1000) or 38% ammonium sulfate precipitated anti-D12 ascites (1:1000), prior to the addition of alkaline phosphatase labelled Goat anti-mouse IgG (Fc specific).

Western blot analyses utilizing a rabbit anti-TNP antibody (not shown), the presence of low concentrations of antigen in the sera binding the 3/B9 idiotype could not be completely ruled out.

3.13 Passive transfer of anti-idiotypic antibodies does not affect the anti-DNP IgE response.

To determine whether or not bracketing the priming immunization (DNP-OA in alum) with anti-idiotypic antibodies against an anti-DNP IgE idiotype could prevent the induction of an IgE response, mice received 100 μg anti-D12 mAb, 12 hours before, and 12 hours after priming with 2 μg DNP-OA in alum. As shown in Table 17, the passive administration of anti-D12 was without effect on the subsequent development of an anti-DNP IgE response.

3.14 Anti-DNP IgG subclass distribution: Increased levels of IgG2a as a result of pretreatment with DNP-BP.

As there was no solid evidence for the existence of auto-anti-idiotypic antibodies in the sera of pretreated and primed mice, and since anti-DNP IgG levels were always high after pretreatment, attention was focused on possible differences in IgG subclass distribution between DNP-BP- and TNP-KLH-pretreated mice. Figure 10 clearly shows that while the predominant anti-DNP isotype in DNP-BP pretreated mice was IgG2a (Figure 10A), IgG1 and IgG2a were evenly represented in mice pretreated with TNP-KLH (Figure 10B).

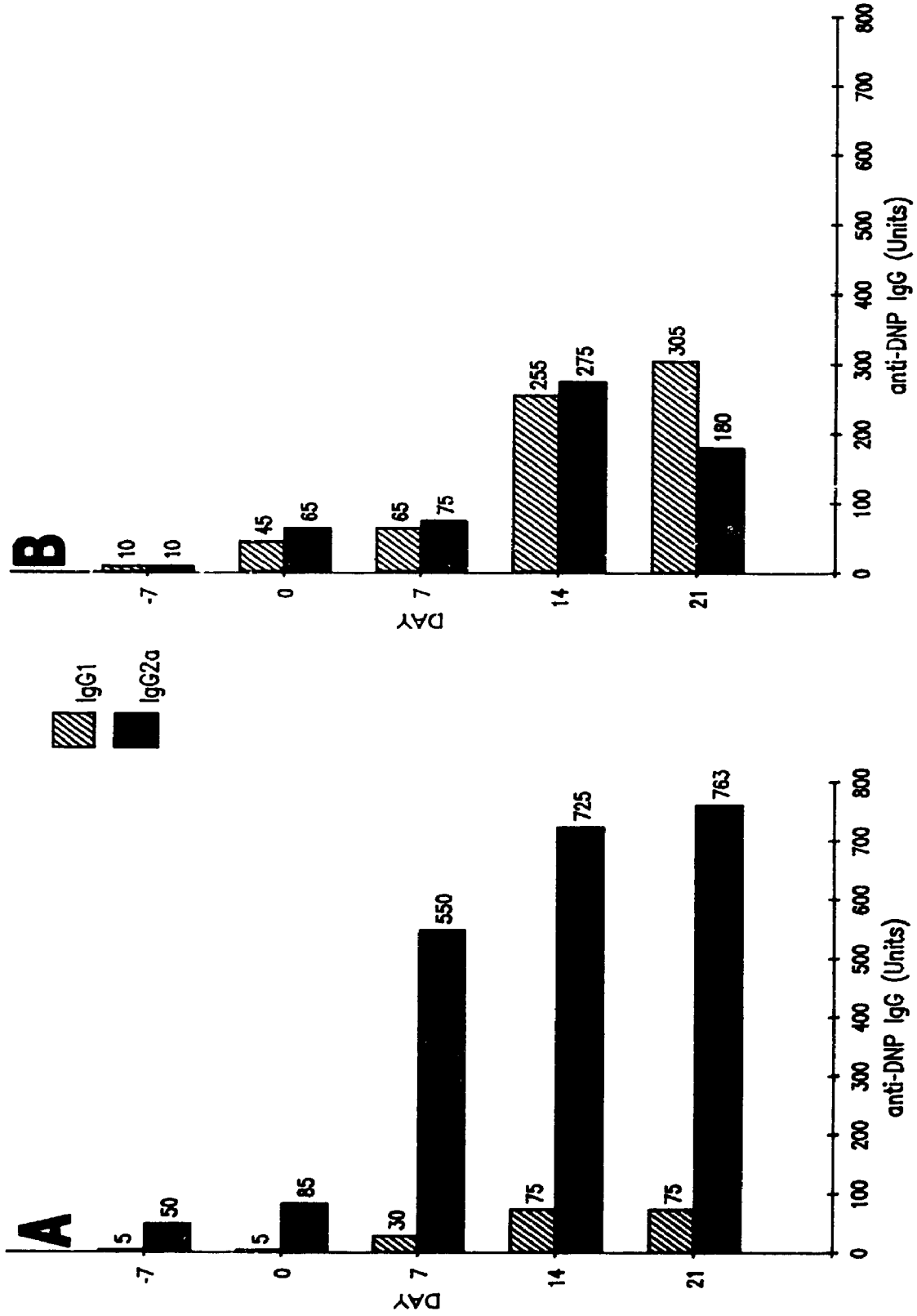
Table 17.

Effects of anti-idiotypic antibody on anti-DNP IgE levels.

Treatment[a]	anti-DNP IgE (PCA titer) day 7	14	21
anti-D12	<20	160	160
anti-NIP	<20	80	160
Saline	<20	160	160

a) Groups of 5 CBA mice received a total of 200 µg of 50% ammonium sulfate precipitated D12 ascites, or DG5.3C12 ascites (anti-NIP IgE), or saline, 12 hours before and 12 hours after immunization with 2 µg DNP-OA in alum.

Figure 10. Distribution of anti-DNP IgG1 and IgG2a isotypes. Groups of 4 mice were treated with 10^9 DNP-BP on days -16, -14 and -12 (A) or with 50 μg TNP-KLH, on the same days (B). The sera obtained on the days indicated were tested for anti-DNP IgG1 (hatched bars) and IgG2a (solid bars) antibodies.


A
B
IgG1
IgG2a
DAY
-7
0
7
14
21
anti-DNP IgG (Units)
0
100
200
300
400
500
600
700
800
5
50
5
85
30
550
75
725
75
763
10
10
45
65
65
75
255
275
305
180

It is interesting to note that the levels of anti-DNP IgG2a were detectable in the DNP-BP pretreated groups already 7 days after DNP-BP injection. In contrast, IgG1 was not detected until 3 days after priming with DNP-OA. For comparison, the anti-DNP IgG subclass distribution after priming with 10^9 DNP-BP or 50 μg TNP-KLH are shown on day 0, just prior to immunization with DNP-OA (Figure 11). When mice were primed with 2 μg DNP-OA in alum only, the levels of anti-DNP IgG1 antibodies were higher than IgG2a (Figure 12).

3.15 IgG subclass shifts as a result of pretreatment with DNP-BP are antigen specific.

As observed with anti-OA PCA titers (Table 4), the IgG1 and IgG2a subclass distribution of anti-OA antibodies was not affected by pretreatment with DNP-BP, and followed the expected pattern of IgG1 and IgE expression observed in mice immunized with DNP-OA in alum alone (Table 18). The fact that the anti-OA IgG1 and IgE levels in mice primed with 2 μg OA-in alum were considerably higher than those of mice primed with DNP-OA in alum, indicated that the predominant antibody response in those mice was against the hapten.

Figure 11. Distribution of anti-DNP IgG1 and IgG2a isotypes 14 days after pretreatment with 10^9 DNP-BP, or 50 μg TNP-KLH. Anti-DNP IgG1 (hatched bars). Anti-DNP IgG2a (solid bars).

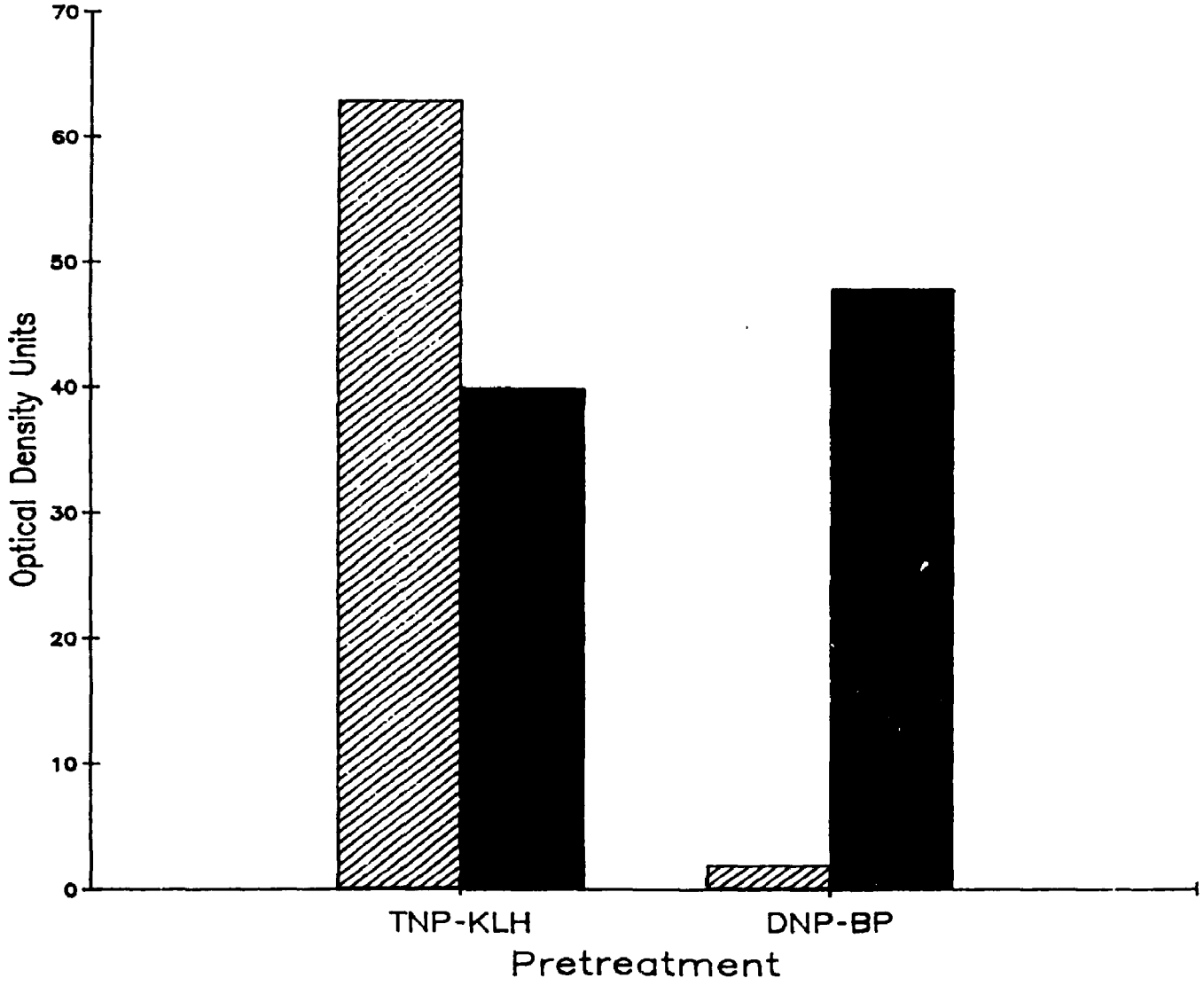

70
60
50
40
30
20
10
0
Optical Density Units
TNP-KLH
DNP-BP
Pretreatment

Figure 12. Distribution of anti-DNP IgG1 and IgG2a as a result of immunization with 2 μg DNP-OA in alum. Anti-DNP IgG1 (hatched bars), anti-DNP IgG2a (solid bars).

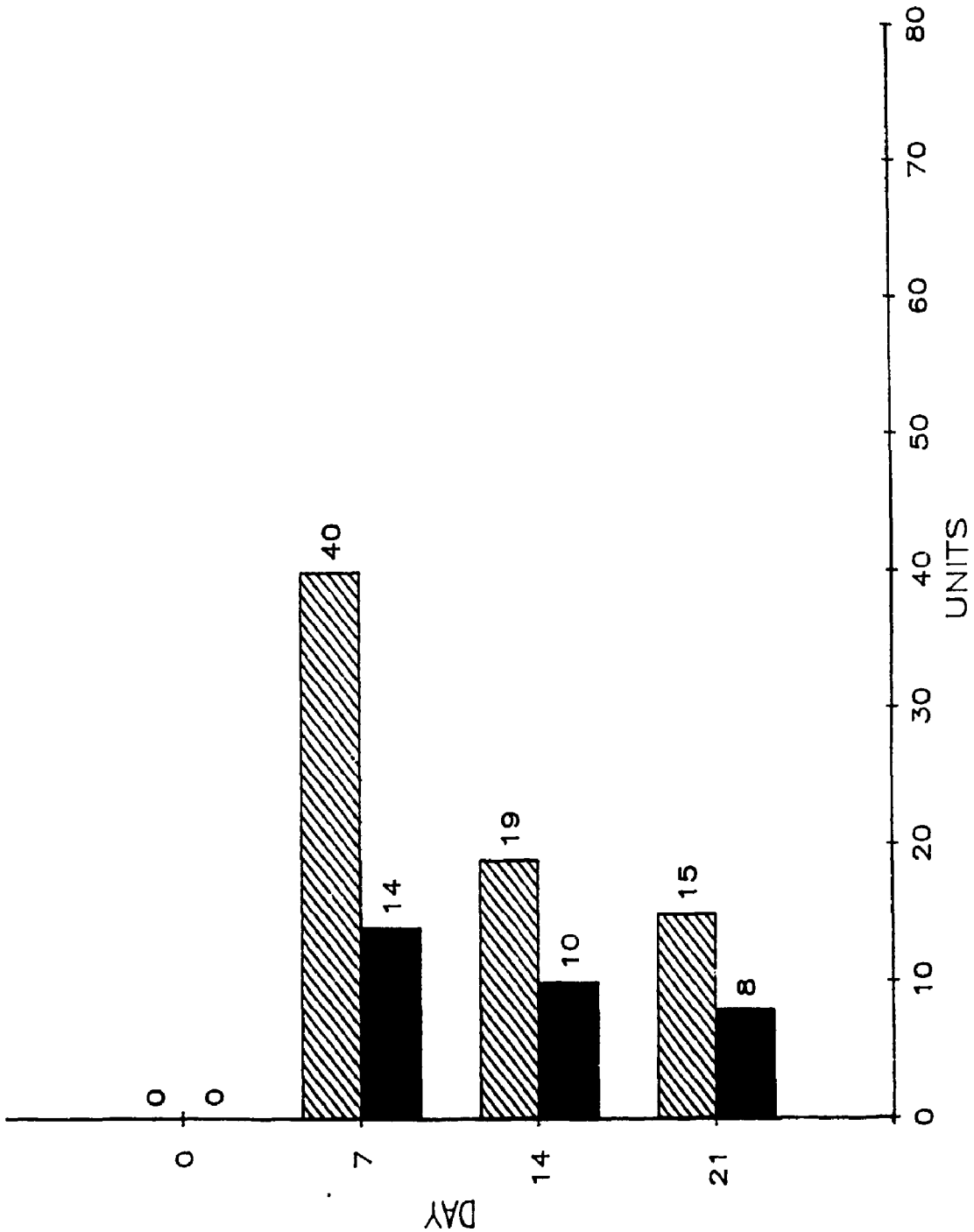

0
0
40
14
19
10
15
8
0
7
14
21
DAY
0
10
20
30
40
50
60
70
80
UNITS

Table 18.

Effect of DNP-BP pretreatment on anti-OA isotype distribution.

Pretreatment[a]	IgG1[b]	IgG2a	IgE[c]
Normal[d]	1	1	0
Saline	10	3	160
TNP-KLH	110	11	160
DNP-BP	110	35	160
None[e]	900	18	400

a) Groups of 4 CBA/J mice were injected with saline, 50 μg TNP-KLH, or 10^9 DNP-BP i.p. on day -14. All mice were immunized with 2 μg DNP-OA in alum on day 0.

b) Anti-OA IgG1 and IgG2a levels were determined on day 14 and are expressed as units/well (see Materials and Methods).

c) Anti-OA PCA titer on day 14.

d) Mice were not immunized.

e) Mice were not pretreated but were injected with 2 μg OA in alum instead of DNP-OA on day 0.

3.16 Effects of DNP-BP on the anti-DNP IgG subclass distribution of an established response.

Immunization of mice with 10^9 DNP-BP, or 50 μg TNP-KLH, 14 days after priming with 2 μg DNP-OA in alum did not have the same effect on the anti-DNP IgG subclass distribution as when mice were pretreated with DNP-BP (Table 19). In this case, all groups had elevated levels of IgG1 7 days after injection. By day 28 (14 days after injection), although the levels of anti-DNP IgG2a had risen significantly, and were actually greater than those of IgG1, they did not predominate. Even after booster, by day 42, the ratio of IgG1 to IgG2a was fairly even in the group pretreated with DNP-BP (Table 19). It was obvious however that DNP-BP was not without effect, since the relative levels of anti-DNP IgG2a in the DNP-BP-pretreated group were significantly higher than in the other two groups of mice.

3.17 Demonstration of interferon-gamma, and its capacity to regulate immunoglobulin isotype expression.

Based on a number of recent publications demonstrating reciprocal isotype-specific regulation by lymphokines (Isakson et al., 1982; Snapper and Paul, 1987), the results described above suggested that the inability of DNP-BP to promote anti-DNP IgE antibody production might have been due to increased production of IFN-gamma.

Table 19.

Effects of DNP-BP on anti-DNP IgG subclass distribution in an established response.

	Units IgG[a]									
	day 14		21		28		35		42	
Trmt.[b]	γ1	γ2a	γ1	γ2a	γ1	γ2a	γ1	γ2a	γ1	γ2a
DNP-BP	nd	nd	335	70	1185	1367	2432	3500	4664	5000
TNP-KLH	nd	nd	1460	520	1090	68	1786	67	1987	109
Saline	14	12	10	8	nd	nd	nd	nd	973	111

a) Levels of anti-DNP IgG1 and IgG2a were determined by EIA. Units refers to the optical density reading obtained having a value of twice background, multiplied by the dilution giving that reading.

b) Treatment of mice. Groups of 5 CBA mice were immunized with 2 μg DNP-OA in alum on day 0. On day 14 they received an i.p. injection of 10^9 DNP-BP, or 50 μg TNP-KLH, or saline. All mice were boosted on day 28 with 2 μg DNP-OA in alum.

i) Viral plaque reduction assay.

Initial attempts to detect IFN-gamma utilized VSV in a viral plaque-reduction assay (see Materials and Methods). The results of those studies indicated that the serum of mice pretreated with DNP-BP reduced the number of viral plaques to a greater extent than the serum of normal or TNP-KLH-pretreated mice (Table 20). The inhibition was sensitive to low pH dialysis, suggesting that IFN-gamma was neutralized. The differences between the groups appeared to be the greatest early during the response, 48-96 hours after pretreatment. Furthermore, culture supernatants of spleen cells from groups of mice primed with DNP-BP also demonstrated elevated levels of pH 2- sensitive interferon as compared to other groups (not shown). This assay was however, very difficult to reproduce from one run to the next, and as judged by titration with an IFN-gamma standard, exhibited low sensitivity (not shown). The problems associated with the viral plaque reduction assay as a test for the measurement of IFN-gamma are not unique to this laboratory (Dr. R. Coffman, personal communication), and may have to do with the fact that IFN-gamma is not an anti-viral lymphokine, but rather a growth-promoting factor for various lymphocytes (Landolfo et al., 1988).

Table 20.

Viral Plaque Reduction Assay: Detection of IFN-gamma.

Treatment[a]	Serum tested[b]	# Plaques/well	% Inhibition[b]
DNP-BP 1x	undialyzed[c]	249 ± 65	43
	dialyzed	429 ± 33	< 1
TNP-KLH 1x	undialyzed	378 ± 0	13
	dialyzed	426 ± 42	2
DNP-BP 2x[d]	undialyzed	237 ± 10	45
TNP-KLH 2x	undialyzed	274 ± 45	37
NMS		404 ± 51	7
IFN-gamma[e]		193 ± 44	55

a) Groups of 4 CBA mice were immunized with 10^9 DNP-BP, or with 50 μg TNP-KLH, at time 0 and at 48 hours. Serum was added to the assay at a 10% concentration. Mice were bled 48 hours after the last immunization.

b) Percent inhibition was based on the number of viral plaques in the absence of serum, 433 ± 35.

c) Sera were tested either undialyzed, or dialyzed against 0.1M Glycine-HCl - 0.15M NaCl pH 2.0, to neutralize IFN-gamma.

d) Mice were bled 96 hours after the first immunization.

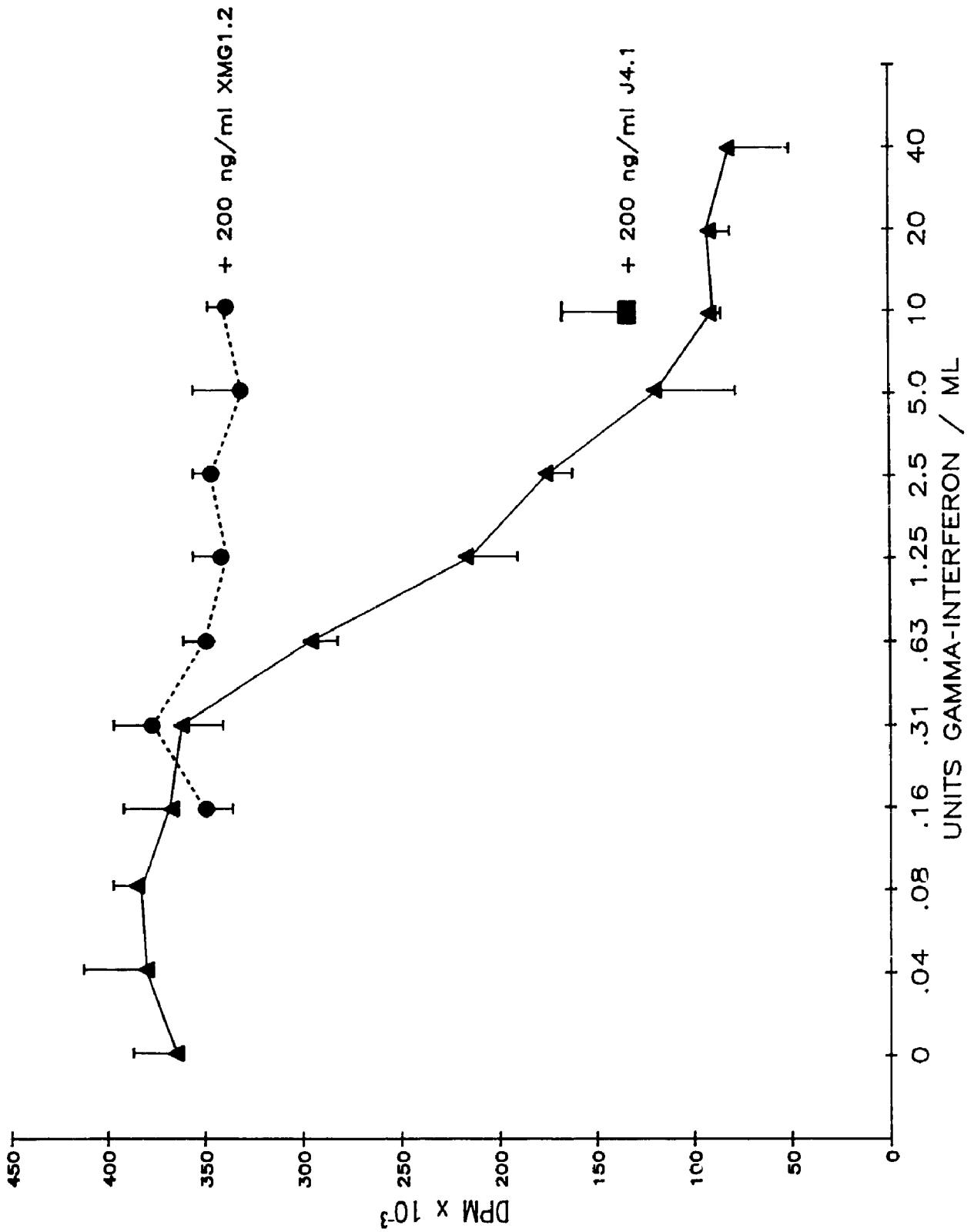
e) 10 Units of recombinant IFN-gamma.

ii) In-vitro bioassay: Detection of IFN-gamma in cell culture supernatants.

As the sensitivity and reproducibility of the viral plaque reduction assay was low, supernatants were tested for IFN-gamma by their ability to inhibit the proliferation of the B-lymphoma WEHI-279. This assay was found to be sensitive to 0.63 U rIFN-gamma/ml (Figure 13).

Spleen cells from mice pretreated with DNP-BP, with TNP-KLH, or from normal mice, were removed 72 hours after injection. They were cultured in the presence or absence of 50 μg/ml (10^9) DNP-BP or TNP-KLH for 36 hours, as shown in Table 21. The presence of IFN-gamma in the culture supernatants was confirmed by the inclusion of 200 ng/ml of a neutralizing anti-IFN-gamma monoclonal antibody (XMG1.2) to a parallel set of supernatants. The results from 2 separate experiments clearly show that the spleen cells of DNP-BP-injected donors released - in the presence of DNP-BP - approximately 1.25 to 5 units IFN-gamma/ml, and that these were completely neutralized by the XMG1.2 antibody. It is interesting to note, that DNP-BP also induced the release of IFN-gamma from normal spleen cells. In one of the two experiments, spleen cells from DNP-BP primed donors released IFN-gamma even in the absence of antigen. Spleen cells from TNP-KLH treated donors released IFN-gamma when incubated with TNP-KLH, but the amounts were much smaller than those released under similar conditions by spleen cells of DNP-BP-pretreated mice. Moreover, these cells did not release IFN-

Figure 13. Titration of recombinant murine IFN-gamma standard on the B lymphoma WEHI-279. Serial dilutions of standard rIFN-gamma in 0.1 ml complete medium were added to wells of a 96-well flat bottomed-plate, containing 10^4 WEHI-279. The mixture was incubated for 72 hours at 37°C. 0.5 uCi/well of ^{3}H-thymidine was added for the last 18 hours. The results are given in disintegrations per minute (DPM) for cells grown in the presence of rIFN-gamma alone (▲), in the presence of rIFN-gamma pre-incubated with 200 ng/ml anti-IFN-gamma mAb, XMG1.2 (●), or pre-incubated with 200 ng/ml anti-NP mAb J4.1 (■). Vertical bars represent standard deviations of triplicate cultures.


+ 200 ng/ml XMG1.2
+ 200 ng/ml J4.1
DPM x 10-3
450
400
350
300
250
200
150
100
50
0
0
.04
.08
.16
.31
.63
1.25
2.5
5.0
10
20
40
UNITS GAMMA-INTERFERON / ML

a) Donor mice were injected i.p. with 50 μg TNP-KLH, 10^9 (50 μg) DNP-BP or were left untreated. After 72 hours the spleens were removed and $4x10^6$ spleen cells/ml were cultured with or without antigen for 24 hours. Cell-free supernatants were removed and tested for their ability to inhibit the proliferation of the B- lymphoma WEHI-279. All supernatants were tested at a final dilution of 1:2.

b) TNP-KLH or DNP-BP were added in culture at a final concentration of 50 μg/ml (10^9 DNP-BP).

c) WEHI-279 cells were cultured in triplicate at 10^4 cells per well in the presence or absence of anti-IFN-gamma antibody XMG 1.2 for 72 hours. ^3H-thymidine was added for the last 18 hours of culture. Values indicate disintegrations per minute x 10^{-3} ± standard deviation of the replicates.

d) Percent inhibition of proliferation induced by supernatants without XMG 1.2 antibody, as compared to proliferation in the presence of supernatants of normal spleen cells grown without antigen.

e) Percent neutralization of inhibitory effect following the addition of 200 ng/ml XMG 1.2.

Table 21.

Effect of spleen cell supernatants on the proliferation of WEHI-279 cells. Evidence for the presence of IFN-gamma.

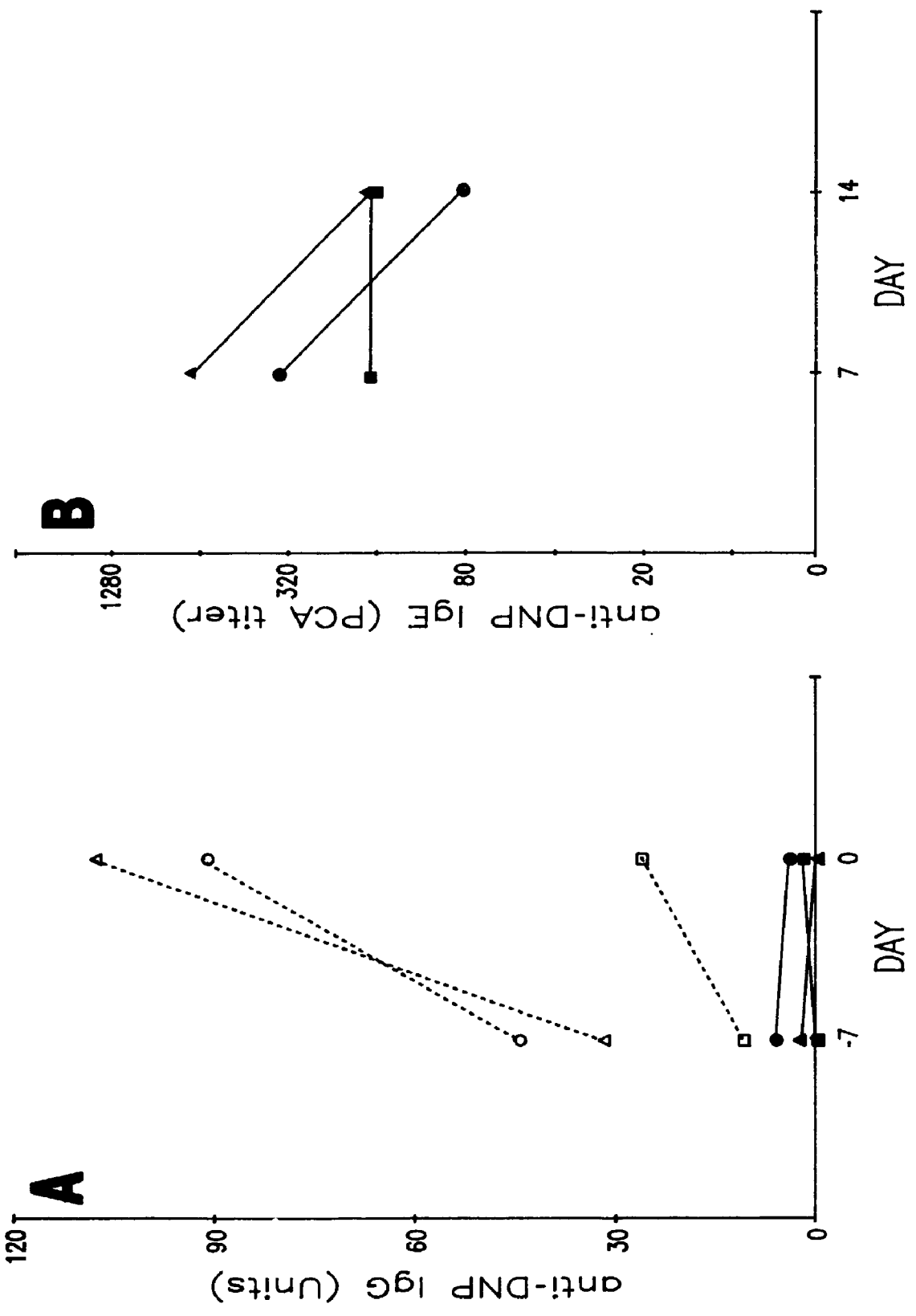
Spleen cells[a]	Antigen[b]	DPM x 10^{-3}[c] No Ab	DPM x 10^{-3}[c] XMG1.2	%Inhibition[d]	%Neutralization[e]
Exp.1					
Normal	None	389 ± 10	344 ± 10	--	--
	TNP-KLH	382 ± 50	369 ± 13	2	107
	DNP-BP	310 ± 8	358 ± 71	20	104
TNP-KLH	None	378 ± 10	378 ± 4	3	110
	TNP-KLH	344 ± 11	334 ± 15	12	97
DNP-BP	None	330 ± 20	376 ± 9	15	109
	DNP-BP	268 ± 5	336 ± 45	31	98
Exp.2					
Normal	None	362 ± 5	349 ± 4	--	--
	TNP-KLH	340 ± 11	365 ± 8	6	105
	DNP-BP	265 ± 25	340 ± 5	27	97
TNP-KLH	None	356 ± 4	364 ± 9	2	104
	TNP-KLH	288 ± 3	360 ± 3	20	103
DNP-BP	None	364 ± 8	363 ± 21	-1	104
	DNP-BP	236 ± 15	334 ± 15	35	96

gamma in the absence of specific antigen, and TNP-KLH was unable to induce IFN-gamma release from normal spleen cells.

iii) In-vivo demonstration of the role of IFN-gamma on the anti-DNP IgE response.

The release of IFN-gamma by spleen cells from DNP-BP-primed donors *in vitro*, suggested that the suppression of the anti-DNP IgE response observed *in vivo*, after the administration of DNP-BP, might also be attributed to IFN-gamma. To test this assumption, three groups of CBA/J mice were pretreated with 10^9 DNP-BP on day -14, and were injected 36 hours later with 1 mg affinity-purified anti-IFN-gamma XMG6, 1 mg affinity-purified anti-NP antibody J4.1, or saline, intravenously. On day 0, all mice were immunized with 2 μg DNP-OA in alum. They were bled on days -7, 0, 7 and 14, and their sera were tested for IgG1, IgG2a, and IgE anti-DNP antibodies. Figure 14 shows that treatment with anti-IFN-gamma determined a drastic decrease in anti-DNP IgG2a levels (Figure 14A), and eliminated the characteristic spike and suppression of PCA titers which was otherwise observed in DNP-BP-pretreated animals after priming with DNP-OA in alum (Figure 14B). The groups that were injected either with 1 mg J4.1, an isotype-matched but irrelevant monoclonal antibody, or with saline, exhibited a 4-fold drop in PCA titers from day 7 to day 14. Anti-DNP IgG1 levels were low in all cases since, as shown in Figure 10A, DNP-BP did not stimulate significant levels of IgG1.

Figure 14. Effects of anti-gamma-interferon antibody on anti-DNP IgG1, IgG2a and IgE production in vivo. Three groups of 4 mice were pretreated with 10^9 DNP-BP on day -14 and primed with 2 μg DNP-OA in alum on day 0. Thirty six hours after DNP-BP pretreatment, separate groups of mice received 1 mg anti-gamma-interferon mAb XMG6 (□ , ■), 1 mg anti-NP mAb J4.1 (△ , ▲), or saline (○ , ●) i.v. A. Anti-DNP IgG1 (closed symbols) and IgG2a (open symbols) are expressed in units. B. Anti-DNP IgE (closed symbols) are expressed as PCA titers.


A
anti-DNP IgG (Units)
120
90
60
30
0
-7
0
DAY
B
anti-DNP IgE (PCA titer)
1280
320
80
20
0
7
14
DAY

3.18 IgG2a does not regulate levels of anti-DNP IgE.

The results described above showed that mice pretreated with DNP-BP and primed with DNP-OA had elevated levels of anti-DNP IgG2a and of IFN-gamma. Since in addition to IFN-gamma, the high anti-DNP IgG2a levels, by themselves, could have been directly involved in the IgE suppression observed, through a feedback regulatory loop, it was deemed important to determine the extent of an anti-DNP IgE antibody response in the presence of anti-DNP IgG2a antibodies produced outside the context of increased IFN-gamma.

Thus, mice were pretreated with TNP-KLH (known not to increase IFN-gamma levels), and $5x10^6$ ascites-grown anti-DNP IgG2a (B4D4), or IgG1 (AC2A12) hybridoma cells were injected i.p. (without pristane preparation), in order to achieve an intravascular concentration of the desired IgG isotype, approximating that occuring after pretreatment with DNP-BP. This approach was taken following preliminary trials which indicated that, in the absence of pristane preparation, hybridomas injected i.p. were well tolerated by the recipients for at least 30 days, during which time their serum contained elevated levels of antibodies exhibiting the specificity and isotype of the hybridoma transferred.

The results in Table 22B show that mice receiving hybridoma cells 7 days after pretreatment with 50 μg TNP-KLH had at the time of immunization with DNP-OA (day 0), elevated levels of the expected anti-DNP IgG isotype. It is evident that anti-DNP IgE titers were not affected by either

a) Groups of 4 CBA mice were immunized with 50 μg TNP-KLH 7 days prior to the transfer of $5x10^6$ ascites grown hybridoma cells. The transferred hybridomas secreted either anti-DNP IgG1 (AC2A12) or IgG2a (B4D4). All mice were challenged with 2 μg DNP-OA in alum 7 days after cell transfer. Mice were bled 7 days after cell transfer (prior to immunization), and at weekly intervals thereafter.

b) Not determined.

c) Levels of anti-DNP IgG were determined by EIA as described at Materials and Methods.

Table 22.

Anti-DNP IgE titers are not affected by anti-DNP IgG subclass concentrations in-vivo.

A.

Cells transferred[a]	anti-DNP IgE (PCA titer)			
	day 0	7	14	21
none	0	320	160	80
AC2A12	nd[b]	160	160	160
B4D4	nd	160	160	160

B.

Cells Transferred	anti-DNP IgG (units)[c]							
	day 0		7		14		21	
	τ1	τ2a	τ1	τ2a	τ1	τ2a	τ1	τ2a
none	75	106	224	136	1946	333	2468	384
AC2A12	212	77	239	120	2085	371	1885	303
B4D4	41	371	264	4710	2540	4014	2458	609

IgG1 or IgG2a at this time (Table 22A).

4. Discussion

The murine IgE antibody response is highly dependent upon a number of factors for its induction. Although the IgE response to protein antigens shares many immunoregulatory mechanisms with other immunoglobulin isotypes, it differs in several aspects. IgE is exquisitely sensitive to both positive and negative T cell regulatory effects, as well as to antibody feedback mechanisms (Katz et al., 1974; Tada, 1975; Ishizaka, 1976; Ishizaka and Ishizaka, 1978; Cvary et al., 1978; Katz 1978; Kishimoto 1982). Critical for the induction of a good IgE response, is the choice of adjuvant administered during immunization. Although complete Freund's adjuvant is an excellent inducer of IgG responses to antigen, it is a poor adjuvant for the induction of IgE responses, due in part to the generation of IgE-selective suppressor factors (Tung et al., 1978; Hirashima et al., 1980; Katz et al., 1980; Jardieu et al., 1985; Akasaki et al., 1986).

Bordetella pertussis vaccine (Mota, 1967; Clausen et al., 1969), and aluminium hydroxide gel (alum) (Revoltella and Ovary, 1969; Levine and Vaz, 1970; Vaz et al., 1971), are very effective at inducing IgE responses. The mechanism by which B. pertussis enhances antibody responses to protein antigens has been attributed to the generation of IgE-selective potentiating factors (Hirashima et al., 1981; Iwata et al., 1983).

The objective of the present investigation was to determine whether immunization of mice with a hapten (DNP) coupled to B. pertussis vaccine would result in an enhanced anti-hapten IgE antibody response. The results of the present study confirmed the initial work carried out by Essani (1983), who showed that CBA mice immunized with DNP-BP either in saline, or adsorbed on alum, failed to produce detectable anti-DNP IgE levels after primary stimulation and only low levels of anti-DNP IgE after booster. The inability of DNP-BP to stimulate IgE production in mice could not be attributed to a lack of immunogenicity of the preparation, since high concentrations of anti-DNP IgG antibodies were produced. Furthermore, DNP-specific IgE-B memory cells could be demonstrated by adoptive transfer.

These results are in complete agreement with those of Essani (1983). In that study, it was also observed that the amount of DNP-BP, or the DNP-to-BP substitution ratio, did not affect the development of the anti-DNP IgE response induced as a result of immunization with DNP-BP. Other studies have shown that the magnitude of an IgE response to antigen varies according to the strain of mouse (Revoltella and Ovary, 1969; Levine and Vaz, 1970; Chiorazzi et al., 1976, 1977; Watanabe et al., 1976). In a detailed analysis of strain responsiveness to immunization with DNP-BP, Essani (1983) clearly demonstrated that mice fell into 2 response patterns. CBA mice (H-2^{k}) were low responders (maximum secondary response PCA titer 1:80), whereas Balb/c mice

($H\text{-}2^a$) were found to be high responders (primary PCA titers 1:100). All strains of mice tested produced excellent anti-DNP IgG levels (Essani, 1983). These results suggested that an active T cell-dependent suppressor mechanism operating in an immunoglobulin class-selective (Watanabe et al., 1976; Chiorazzi et al., 1976, 1977) or non-selective fashion (Kapp et al., 1974) might be involved. However, the low levels of in situ IgE expression in CBA mice could not be enhanced by treatment with low doses of x-irradiation, or of cyclophosphamide (Essani, 1983), indicating that the suppression was not operating through mechanisms previously described (Watanabe et al., 1976; Chiorazzi et al., 1977).

The fact that DNP-BP induced anti-DNP-IgE B memory cells was, however, indicative of its stimulatory capacity. Indeed, the transfer of DNP-BP-primed spleen cells to sublethally irradiated recipients together with OA-primed spleen cells prior to challenge with DNP-OA in alum, resulted in a secondary anti-DNP-IgE response (PCA titer 1:1600). These results are similar to those obtained by Hamaoka et al., (1973; 1974) and Katz et al. (1974), who demonstrated that although mice immunized with antigen in CFA did not produce IgE in situ, they did develop IgE B-memory cells, demonstrable after adoptive transfer with appropriate T-helper cells.

In this study, pretreatment of mice with DNP-carrier conjugates followed by immunization with DNP-OA in alum led to the appearance of 2 distinct response patterns, depending

on the nature of the carrier used for pretreatment (Figure 4). When CBA mice were pretreated with DNP-BP, and immunized with DNP-OA in alum two weeks later, they displayed a peculiar anti-DNP IgE response pattern that was characterized by an accelerated primary response on day 7, followed within 72 hours by a drastic decline. Similar pretreatment with other DNP-carrier, or TNP-carrier conjugates, also resulted in an accelerated primary anti-DNP IgE response following immunization with DNP-OA, however, PCA titers did not decline. Pretreatment with saline, or unsubstituted BP, resulted in the development of a normal primary anti-DNP IgE response after immunization with DNP-OA in alum.

These results suggested that the suppressive mechanism operating in mice pretreated with DNP-BP was different from the one operating in mice primed and boosted with DNP-BP. In the latter situation, mice never developed significant levels of anti-DNP IgE (Figure 1). The reason why the homologous (DNP-BP) system in CBA mice was so effective in exerting its suppressive effect on IgE production is not clear, but could have operated through the generation of carrier (BP)-specific T suppressor cells selective for IgE, or through feedback suppression by large amounts of anti-carrier IgG antibody. These potential mechanisms cannot however explain the suppression of the response induced as a result of priming with DNP-BP and immunization with DNP-OA, since different carriers were used for pretreatment (BP) and

for priming (OA). Therefore, anti-carrier IgG antibodies, or putative carrier (BP)-specific T suppressor cells would not be recalled by priming with DNP-OA. In this regard, it is interesting to note that the suppressive event induced by DNP-BP was strictly dependent upon the linked recognition of the DNP-BP conjugate (Table 1). Mice pretreated with 10^9 BP mixed with 50 μg TNP-KLH did not develop the day 7 spike and suppression characteristic of DNP-BP pretreatment. This observation suggests that the induction of the suppressive mechanism may be a complex multi-step event, requiring BP for its induction, and DNP (the antigen) for its specificity. It was conceivable that the effects of the DNP-BP conjugate were mediated on different cell types, and that the suppression observed 72 hours after the anti-DNP IgE spike was the end result of the interaction of a variety of cells and their products.

The delayed suppression observed in mice pretreated with DNP-BP and immunized with DNP-OA was hapten-specific, since the anti-OA IgE antibody response to immunization with either OA or DNP-OA was not suppressed. Indeed, even pretreatment with NIP-BP prior to immunization with NIP-DNP-OA was without suppressive effects on the anti-DNP IgE response, clearly demonstrating the hapten specificity of the suppressive mechanism. Collectively, these results precluded any similarity between the suppressive mechanism observed in this study, and that operative in mice immunized with DNP-Mycobacterium (Kishimoto et al., 1976, 1978, 1982).

Although the suppression initiated by DNP-Mycobacterium was also capable of restimulation by DNP on a heterologous carrier, it appeared early, was antigen-nonspecific, and was dependent on the presence of T suppressor cells (Shiho et al., 1982).

The suppressive mechanisms described by several laboratories share the common characteristics of being antigen-nonspecific and occurring naturally in low IgE responder strains (Watanabe et al., 1976; Chiorazzi et al., 1976, 1977). Furthermore, they are T cell-dependent in that they are transferable by T cells, and low doses of x-irradiation or pretreatment with cyclophosphamide can abrogate the suppression (Watanabe et al., 1976; Chiorazzi et al., 1977). In general, these mechanisms are induced by the administration of adjuvants containing Mycobacterium in a water-in-oil emulsion (CFA)(Kishimoto et al., 1978; Katz and Tung, 1978; Hirashima et al., 1982). The serum, cell-free supernatants, and T-T cell hybrids derived from the lymphoid cells of CFA- or hapten-Mycobacterium-treated animals contained, and released, soluble molecules that were very effective in the suppression of both in vivo and in vitro IgE responses (Suemura et al., 1977; Tung et al., 1978; Hirashima et al., 1981; Shiho et al., 1982). In many situations, both IgE-suppressor and IgE-enhancing molecules have been shown to coexist in vivo, their individual presence demonstrable only after separation by affinity chromatography on lectin-conjugated insoluble matrices (Katz

et al., 1979; Hirashima et al., 1981).

The suppression observed following pretreatment of mice with DNP-BP and immunization with DNP-OA in alum does not seem to fit into a recognizable pattern described for either CFA or for DNP-Mycobacterium. On the contrary, it appears to be unique in several respects, including its induction by an adjuvant known to potentiate IgE responses, its specificity for hapten in terms of both its induction and effect, and its inability to be transferred by lymphoid cells to naive recipients.

A feature common to the immunoregulation of all isotypes, is their sensitivity to the effects of different subpopulations of T cells. It is well established that suppressor T cells can downregulate immune responses (Gershon and Kondo, 1971; Gershon, 1974; Basten et al., 1977). The down-regulation of IgE antibody production by suppressor T cells has been demonstrated in numerous instances (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). In general, the potential for T cell mediated suppression has been demonstrated by the adoptive transfer of spleen cells from suppressed mice into syngeneic recipients (Okumura and Tada, 1971; Kishimoto et al., 1976; Takatsu and Ishizaka, 1976; Ovary et al., 1978; Schwenk et al., 1979).

The results of this study, and that of Essani (1983), found no evidence for the existence of T suppressor cells by adoptive transfer (Table 6,7), implying that suppressor T cells were not directly involved in the suppression observed after pretreatment with DNP-BP and immunization with DNP-OA in alum.

The fact that the anti-DNP IgE levels appeared in an accelerated but transient fashion in the mice pretreated with DNP-BP and immunized with DNP-OA in alum, suggested that an active mechanism of suppression was responsible for the sharp decline in IgE levels after the day 7 spike. Indeed, the transfer of spleen cells from mice previously immunized with DNP-OA in alum into DNP-BP-pretreated mice, prior to immunization with DNP-OA in alum, resulted in the development of an IgE response characterized by a day 7 spike followed by suppression (Table 3). The importance of the DNP-BP-pretreated environment was also evident when spleen cells were transferred from DNP-BP-pretreated mice into normal or sublethally irradiated recipients. Recipient mice developed normal anti-DNP IgE titers in response to immunization with DNP-OA in alum. This implied that the suppressive mechanism could not be transferred directly with spleen cell populations, and that perhaps an intact splenic architecture was necessary for suppression. Alternatively, certain arms of the suppressor network may reside outside of the spleen, or, the presence of the molecules responsible for suppression may be required at the time of IgE B cell

development, at a concentration not maintained after the transfer of a limited number of lymphoid cells.

The suppression induced following immunization with DNP-OA in alum of DNP-BP-pretreated mice, was always associated with high levels of anti-DNP IgG at the time of immunization with DNP-OA in alum (Figures 3,4). This observation suggested that suppression might be the result of an antibody-mediated feedback mechanism by anti-DNP IgG, similar in nature to that described in other systems (Rawley and Fitch 1964; Uhr and Moller, 1968; Sinclair, 1969). Indeed, the passive transfer of unabsorbed serum from DNP-BP/DNP-OA-treated mice profoundly suppressed the primary anti-DNP IgE response of recipients primed with DNP-OA in alum (Table 8). This suppression was found to depend on the concentration of anti-DNP IgG antibody transferred.

Immunoregulation of IgE antibody production by antibody feedback has been shown to occur in mice. The passive transfer of anti-OA IgG antibodies to mice suppressed the induction of both IgE and IgG antibodies against that antigen. It did not however terminate preexisting antibody formation (Ishizaka and Okudaira, 1972). The transfer of anti-DNP IgG antibody into mice pretreated with TNP-KLH and primed with DNP-OA in alum, or into mice immunized with DNP-OA in alum alone, did not affect an established anti-DNP IgE response (not shown).

In the DNP-BP/DNP-OA system, there appears to be a clear delineation between the anti-DNP IgE response and the anti-DNP IgG response. Although the IgE response to the hapten decreases dramatically within 72 hours of the day 7 spike, anti-DNP IgG levels continue to increase. Furthermore, groups of mice pretreated with TNP-KLH or DNP-Asc also produced significant levels of anti-DNP IgG in response to pretreatment. Therefore, if IgG against the hapten does feedback inhibit the anti-DNP IgE response in situ, it does not do so in a direct fashion, and operates under a mechanism different from the one described by Ishizaka and Okudaira (1972).

As the DNP-BP-induced suppression always followed the day 7 anti-DNP IgE spike, it was hypothesized that IgE might feedback inhibit its own response against the hapten. The results of the passive administration of a monoclonal anti-DNP IgE antibody mixture showed that this was not the case (Table 9). Indeed, if anti-DNP IgE inhibited the response, then one would have expected to see a decline in the in situ anti-DNP IgE level of mice receiving the anti-DNP IgE monoclonal antibody mixture.

Although the above observations are in agreement, or are consistent, with those of Essani (1983), the ability of DNP-depleted serum from DNP-BP/DNP-OA-treated mice to suppress the response of recipients after transfer was not reproducible. This suggests that the suppression observed by Essani (1983) was probably due to the incomplete

depletion of anti-DNP antibody from absorbed sera.

Based on results demonstrating that passive transfer of anti-DNP-depleted sera suppressed the anti-DNP IgE response of recipients, that the suppressive activity eluted with the bulk of immunoglobulins after gel filtration, and that no antibody-feedback regulation could be implicated, Essani (1983,1984) proposed that the mechanism involved in the suppression of the anti-DNP IgE response was due to the presence of auto-anti-idiotypic antibodies in the sera of DNP-BP/DNP-OA-treated mice. This proposed mechanism was supported by results obtained by Blaser et al. (1980), who demonstrated that IgE antibody production was more susceptible than IgG to regulation by anti-idiotypic antibodies. Those results correlated well with the observation that anti-DNP IgE, and not IgG, was affected by pretreatment with DNP-BP. Indeed, immunoregulation of the IgE response by anti-idiotypic antibodies was demonstrated by Blaser et al. (1980), and by Blaser and de Weck (1982), for various haptens, and for Timothy grass pollen by Malley and Dresser (1983).

It was reasoned that a significant portion of the anti-DNP antibodies produced in response to DNP-BP/DNP-OA immunization may share a common or cross-reactive idiotype, such as those found in the mouse anti-DNP myelomas MOPC-315 or MOPC-460 (Eisen et al., 1968; Zeldis et al., 1979; Dzierzak et al., 1980). The decrease in the titers of anti-DNP IgE might then be due to anti-idiotypic antibodies

directed against a few predominant idiotypes shared between IgG and IgE anti-DNP antibodies.

The demonstration of an immunoregulatory role for anti-idiotypic antibodies depends upon the ability to detect and quantitate idiotype-anti-idiotype interactions. In the case of binding-site-directed anti-idiotypic antibodies, the detection and quantitation can be based on the ability of the anti-idiotype to inhibit the binding of radiolabelled antigen to the idiotype (Geha, 1982; Bose et al., 1984).

Serum depleted of DNP-specific antibody (the idiotype) by absorption over DNP-Sepharose, bound to and inhibited the reaction of anti-DNP antibody (affinity-purified idiotype) with antigen (DNP) in a concentration-dependent fashion (Essani, 1983; Essani et al., 1984). The fact that the inhibition was abrogated in the presence of unabsorbed serum where the idiotype was in excess, but not in the presence of normal serum, seemed to support the presence of anti-idiotypic antibodies in the serum of mice.

Although the above results implicated the existence of auto-anti-idiotypic antibodies in the serum of mice suppressed as a result of pretreatment with DNP-BP and immunization with DNP-OA in alum, appropriate controls were not included in the aforementioned study (Essani, 1983; Essani et al., 1984). The present study showed that aqueous solutions passed through various types of antigen-coupled immunosorbent matrices, acquired antigen that was capable of inhibiting the binding of radioactively labelled antigen

with its specific antibody. The inhibition was demonstrable in both solid-phase and fluid-phase radioimmunoassays, as well as by EIA (Hagen and Strejan, 1987).

The detection and quantitation of site-directed auto-anti-idiotypic antibodies based on the inhibition of binding of radioactively labelled antigen to idiotype, employs extremely small amounts of labelled antigen (200 ng/ml). It takes an equivalent amount of antigenic material released from affinity columns to inhibit the binding of the labelled antigen by 50%. In situ, one must assume that a large proportion of idiotype and anti-idiotype are in complex form. Therefore, to demonstrate putative anti-idiotypic antibody in the sera of mice, one must displace the idiotype from the anti-idiotype. This can be accomplished by the passage of sera over an antigen-immunosorbent (Shepherd et al., 1981). The rationale behind this approach assumes that the affinity of the idiotype for antigen is greater than that of the idiotype for its corresponding anti-idiotype. In order not to interfere with the assay, the absorbed sera should preferably contain no antigen, or at least not more than 1-2% of the amount used in the assay (2-4 ng/ml). The results of the radiolabelled OA-Sepharose leakage study indicated however, that the preparations tested in these experiments released approximately 200-400 ng/ml of detectable antigen (Figure 6).

Normal sera, or sera obtained from mice pretreated with DNP-BP and immunized with DNP-OA in alum, contained significant amounts of DNP when passed over DNP-immunosorbents (Table 10). The amount of antigen leaking from matrices was not significantly affected by conditions such as the duration of exposure to the matrix, temperature, or lack of mechanical agitation (rolling, shaking). Using 200 ng/ml (20 ng/well) of ^{125}I-DNP-BSA, the range of inhibition was 30-70%. This inhibition was dependent on the dilution of the immunosorbent-passed effluent only, and not on the amount of antibody used to coat the plate (Hagen and Strejan, 1987). Furthermore, inhibition was not observed with solutions passed over irrelevant antigen-coupled matrices. Therefore, the evidence in support of the existence of auto-anti-idiotypic antibodies in the serum of DNP-BP-pretreated mice immunized with DNP-OA became questionable.

Since the antigens used in the present experiments differed significantly in size from the putative anti-idiotypic antibodies, it was reasoned that gel filtration might lead to the separation of leaked antigen from immunoglobulin. It was hoped that this separation would enable absorbed sera to be used in the inhibition assays, free of contaminating antigen. The results showed however, that gel filtration of effluents from DNP-BSA-Sepharose passed normal mouse serum, through Sephacryl S-300, a matrix separating protein molecules in the range of 10^4 - $1.5x10^6$

Da, yielded fractions which contained inhibitory molecules over a broad range of molecular weights, including the immunoglobulin and albumin peaks (Figure 7). This indicated that the antigen released from the immunosorbents existed as a broad spectrum of molecular sizes. The absence of inhibitory activity in fraction IV can be attributed to the fact that all fractions were concentrated and dialyzed prior to the assay, and that some of the antigenic fragments were smaller than 12 kDa (the cutoff point of the dialysis bag).

The instability of antigen-coupled Sepharose immunosorbents has been reported by others (Sica et al., 1973; Parikh et al., 1974). Indeed, previous studies have attempted to reduce the amount of leakage by protein-coupled immunosorbents through the stabilization of such matrices with glutaraldehyde (Kowal and Parsons, 1980), or through the preparation of carbonyldi-imidazole (CDI) matrices (Hearn and Harris, 1981). Therefore, in an attempt to reduce the amount of antigen leakage, the immunosorbent matrices used in this study were crosslinked with glutaraldehyde. Neither stabilization with glutaraldehyde, nor the use of a CDI-activated matrix (Reacti-Gel 6X), prevented antigen leakage (Tables 11, 12). Indeed, the reduction in the extent of antigen released was insignificant, and in the case of affinity-adsorbent silica, the release was even more extensive than with Sepharose.

The results of testing a number of immunosorbent matrices, indicated that two types released negligible traces of reactive antigen: matrices coupled with monovalent hapten such as DNP-Lysine, and protein antigens cross-linked by polymerization with glutaraldehyde. Although the matrices coupled with monovalent hapten released significant amounts of hapten as detected spectrophotometrically, it was readily removed by dialysis and did not inhibit the binding of radio-labelled antigen to antibody. The stability of the glutaraldehyde-polymerized antigens was superior to all other matrices tested, as there was no evidence for the presence of antigen in absorbed effluents. However, the elution of antibody from these polymers was extremely difficult, and they were not suitable for use as idiotype in detection assays.

In an attempt to resolve the problems associated with the demonstration of auto-anti-idiotypic antibodies in the serum of suppressed mice, sera were absorbed on monovalent hapten-coupled immunosorbent matrices. The results obtained after such treatment revealed that sera so absorbed were no longer capable of inhibiting the binding of radiolabelled antigen to affinity-purified anti-DNP antibody (Table 13). Thus, it was apparent that the inhibition observed in previous assays was indeed the result of antigen leakage during absorption.

The lack of inhibition by absorbed sera of suppressed mice did not disprove the existence of auto-anti-idiotypic

antibodies. It was still possible that the proportion of idiotype, binding the putative anti-idiotype in affinity-purified antibody preparations, was not sufficient to demonstrate a reduction of antigen binding in the inhibition assay.

Therefore, in an attempt to enrich for a potential predominant idiotype in mice pretreated with DNP-BP and primed with DNP-OA in alum, spleen cells were fused with the myeloma SP2/0. Fusions were also performed at the time of maximal anti-DNP IgE titers, in the hope of obtaining one or more anti-idiotypic hybridomas induced as a result of high idiotypic antibody levels. This procedure seemed feasible, since auto-anti-idiotypic antibody forming cells had been detected in the spleens of mice infected with Schistosoma mansoni (Powell and Colley, 1985), and had been reported in the spleens of mice immunized with TNP-Ficoll (Goidl et al., 1979). Indeed, in the TNP-Ficoll system, it was demonstrated that pulsating of spleen cells with monovalent hapten could increase the number of anti-TNP plaque forming cells, suggesting that anti-idiotypic antibodies were responsible for PFC inhibition (Schrater et al., 1979).

Although we were unable to generate anti-idiotypic hybridomas from Balb/c or CBA mice, a number of anti-DNP IgG and IgE hybridomas were obtained from several fusions. The utilization of these hybridomas to detect anti-idiotypic antibodies in the serum of mice suppressed as a result of DNP-BP/DNP-OA treatment was also without success. In both

EIA and RIA analyses, as well as by Western blotting, evidence for the existence of a predominant idiotype could not be substantiated. Even though the assays used in this study were capable of detecting the interaction of idiotype and deliberately induced anti-idiotypic antibodies, absorbed sera did not bind any of the idiotypes isolated (Tables 13,15,16). The failure to detect a predominant, or cross-reactive idiotype in the sera of mice treated with DNP-BP/DNP-OA, fits with previous observations suggesting that the anti-DNP antibody response is idiotypically heterogeneous (Askonas et al., 1970).

In addition to the failure to detect the presence of a predominant idiotype, or of auto-anti-idiotypic antibodies in the serum of mice, we were also unsuccessful in demonstrating an immunomodulatory effect by passive transfer of a monoclonal anti-IgE idiotype (anti-D12)(Table 17). As mentioned above, it was reasoned that since the anti-DNP IgE response always declined within 72 hours of peak titers, this "spike" may have induced the suppressive event. As the D12 monoclonal antibody was one of only two anti-DNP IgE hybridomas obtained from a fusion of spleen cells of mice immunized and boosted with DNP-OA in alum, it was not unreasonable to assume that this might represent a predominant idiotype.

Taken collectively, the results suggested a number of possibilities regarding the existence, and immunoregulatory role, of auto-anti-idiotypic antibodies in the DNP-BP/DNP-OA system: 1) Although the assay systems employed in this study were capable of detecting the interactions of idiotype and anti-idiotype, their sensitivity was too low to detect their presence in the sera of mice; 2) Shifts in the idiotypic repertoire prevented the detection and isolation of a predominant idiotype (Kelso et al., 1980; Reth et al., 1981; Kim, 1982); 3) The mechanism involved in the suppression of the anti-DNP IgE response did not depend on an anti-idiotypic mechanism.

As the transient expression of anti-DNP IgE antibody occuring as a result of pretreatment with DNP-BP/DNP-OA could not be accommodated by any of the mechanisms known to regulate IgE production, other possibilities were explored.

Recent developments in several laboratories have shown that the IgE response was highly susceptible to the regulatory influences of lymphokines produced by T helper cell subpopulations. These lymphokines are released as a consequence of the interaction between T cells and MHC-linked antigen on the surface of antigen presenting cells (APC). The existence of distinct T helper cell populations was proposed by Janeway (1975), and Marrack and Kappler (1975). With the isolation and generation of clonal T helper cell populations, it was possible to examine the functional properties of these cells. After the examination

of a number of mitogenically activated helper T cell clones, Mosmann et al. (1986) proposed the existence of two distinct subsets. T_H1 were identified through their ability to produce and release, into culture supernatants, IFN-gamma and IL-2 amongst a number of other lymphokines (Mosmann and Coffman, 1987). This subset correlated with the inflammatory subset of helper cells described by Kim et al. (1985). T_H2, were identified by their capacity to provide antigen-specific help to B cells, and their production (amongst other lymphokines) of IL-4 (Kim et al., 1985; Mosmann and Coffman, 1987; Bottomly, 1988), and IL-5 (Mosmann and Coffman, 1987). It is the production and release of IFN-gamma, and IL-4, by these separate helper cell subpopulations that controls B cell isotype expression (Coffman et al., 1988).

In vitro, in LPS-stimulated B cell cultures, IFN-gamma enhances switching from IgM to IgG2a, and inhibits the IL-4-induced switch to IgE and IgG1 (Coffman and Carty, 1986; Snapper and Paul, 1987). Conversely, IL-4 promotes IgG1 and IgE (Isakson et al., 1982; Paul and Ohara, 1987), and inhibits the IFN-gamma-induced switch to IgG2a (Coffman and Carty, 1986; Snapper and Paul, 1987). Examination of a panel of antigen - or Con-A - activated T_H1 and T_H2 clones, indicated that supernatants from T_H1 clones enhanced IgG2a and inhibited T_H2-dependent IgG1 and IgE enhancement of LPS-stimulated B cell cultures. This activity could be blocked by anti-IFN-gamma antibody (Coffman and Carty, 1986; Mosmann

and Coffman, 1987).

As the suppression observed in the current study was always associated with elevated levels of anti-DNP IgG, it was felt that an examination of the anti-DNP IgG isotype profile might provide insights as to whether lymphokines were involved in the regulation of the DNP-BP/DNP-OA immunized mice. The results clearly showed that mice treated with DNP-BP developed significantly higher levels of anti-DNP IgG2a than did mice injected with TNP-KLH (Figures 10, 11). This observation is consistent with those of other recent studies, which have shown that the administration of bacterial vaccines such as Salmonella typhimurium (Duran and Metcalf, 1987) and Brucella abortus (Finkelman et al., 1988) also preferentially stimulated the production of IgG2a antibody.

Further analysis demonstrated that spleen cell cultures taken from mice 72 hours after a single injection of 10^9 DNP-BP released between 1.25 and 5 units/ml of IFN-gamma, when grown for 36 hours in the presence of DNP-BP (Table 21). It must be pointed out that normal spleen cells grown for 36 hours in the presence of DNP-BP also released detectable (albeit smaller) amounts of IFN-gamma. Spleen cells from TNP-KLH- injected mice also produced IFN-gamma, but the amounts released were consistently smaller than those from DNP-BP-primed donors. In fact they were smaller even than those released by normal spleen cells grown with DNP-BP).

The ability of TNP-KLH to trigger the release of IFN-gamma is not surprising since pretreatment of mice with TNP-KLH induced both IgG1 and IgG2a antibody, suggesting that TNP-KLH can induce both T_H1 and T_H2 cells (Figures 10,11). This observation is also consistent with recent reports that both T_H1 and T_H2 clones can be derived from KLH-immunized mice, and that these clones provide help to DNP-specific B cells *in vitro*, according to their predicted lymphokine profile (Cherwinski *et al.*, 1987; Stevens *et al.*, 1988).

The fact that DNP-BP can trigger IFN-gamma release in normal spleen cell cultures suggests that exposure of lymphocytes to DNP-BP, either *in vivo* or *in vitro*, leads to the activation of BP-specific T_H1 cells. The rapidity by which IFN-gamma is released by normal spleen cells in the presence of DNP-BP suggests that BP exerts an antigen-nonspecific (polyclonal) activating effect on a preexisting T_H1 cell pool. Another possibility is that a mechanism of selection of helper T cells expressing antigen-receptor V genes specific for epitopes on common pathogens may have evolved as a consequence of repeated exposures to bacterial infections. Alternatively, low levels of IFN-gamma expression may have been induced by Pertussis toxin (a protein component of *B. pertussis*), which has been shown to induce the release of gamma-interferon through the stimulation of $Lyt2^+$ spleen cells (Vogel *et al.*, 1985).

In order for the above findings to be biologically relevant, a causal relationship between the DNP-BP-dependent induction of IFN-gamma and the decrease in anti-DNP IgE levels must be demonstrated. Indeed the administration of anti-IFN-gamma monoclonal antibody (XMG6) to DNP-BP-treated mice resulted in the abrogation of the IgE decline, since the accelerated (day 7) anti-DNP-IgE response was not followed by a decrease in PCA titers on day 14 (Figure 14). The day 7 PCA titers of this group were lower than those of the groups injected with an irrelevant isotype-matched antibody (J4.1) or with saline. It is important to note however, that while the PCA titers in the two control groups decreased 4 fold from day 7 to day 14, the PCA titers of the group receiving anti-IFN-gamma antibody remained unchanged. This group also showed markedly decreased anti-DNP IgG2a levels. Anti-DNP IgG1 antibodies were low in all groups pretreated with DNP-BP (Figures 10, 11) and no change was detected as a result of anti-IFN-gamma treatment.

The results of the in vivo manipulation of IFN-gamma and its effects on the expression of immunoglobulin isotypes, suggests that the mechanisms of antigen-non-specific isotype regulation described by Finkelman et al. (1986; 1987; 1988) may also be operative in DNP-BP/DNP-OA treated mice. Indeed, Finkelman et al. (1986) clearly demonstrated that the administration of an anti-IFN-gamma monoclonal antibody, together with a polyclonal T and B cell stimulus (anti-IgD antibody), resulted in the production of

increased levels of antigen-non-specific IgE and IgG1.

IFN-gamma exhibits a broad spectrum of immunoregulatory functions. Among these is the ability to activate macrophages and to induce the expression of MHC class II molecules on the surface of antigen-presenting cells (Beller and Unanue, 1981; Wong et al., 1984). The drastic reduction in IgG2a (the predominant isotype produced in response to DNP-BP stimulation, and the slight reduction of anti-DNP IgE on day 7 may be a consequence of deficient antigen presentation by macrophages. Alternatively, anti-IFN-gamma may be involved in preventing activation of T_H cells or in the generation of Lyt2+ T suppressor cells as described by others (Frasca et al., 1988).

The kinetics of the IgE spike and suppression as well as the strict hapten specificity of the phenomenon suggest that the administration of DNP-BP preferentially induces BP-specific T_H1 cells. These release IFN-gamma which in turn promotes the switch of B cells to IgG2a antibody production. It has recently been demonstrated that T_H1 clones, through IFN-gamma, can drastically suppress the antigen or anti-CD3-induced activation of T_H2 clones. This has given rise to the suggestion that T_H1 and T_H2 cells are mutually regulatory subsets (Fernandez-Botran et al., 1988; Gajewski and Fitch, 1988). It can be assumed therefore, that the in vivo isotype profile would ultimately depend on the relative proportion of T_H1 and T_H2 cells generated during antigenic stimulation.

B. pertussis vaccine apparently has two antagonistic functions amongst its biologic properties (Munoz, 1964): one is to potentiate IgE responses (Munoz *et al.*, 1981), the other is to preferentially induce T_H1 cells, IFN-gamma, and IgG2a production. When BP is used as an adjuvant i.e. mixed, but not physically linked to a soluble antigen, the generation of T_H1 cells would dictate the appearance of BP-specific IgG2a and suppressed BP-specific IgE antibodies; this apparently does not interfere with the ability of BP to potentiate T_H2-dependent IgE production directed against the soluble antigen in the mixture. When the antigen is coupled to BP, most of the antigen-specific responses are dictated by the collaboration of BP-specific T_H1 cells with DNP-binding B cells, through linked recognition. Thus, most of the DNP-specific B cells switch to IgG2a. The demonstrated presence of anti-DNP IgE B cells (Figure 2), and the appearance of an accelerated anti-DNP IgE response on day 7 after booster with DNP-OA in alum (Figures 3,4) suggest however, that a small contingent of T_H2 cells are also produced. Indeed, the results of Okudaira and Ishizaka (1973) indicate that antigen-specific IgE B cells appear prior to the formation of IgG producing cells. Thus, early arising DNP-specific IgE B cells may become rapidly activated following booster with DNP-OA in alum which presumably stimulates OA-specific T_H2 cells preferentially.

As discussed above, the suppression of the anti-DNP IgE response which followed the day 7 "spike" could be explained in several ways. The results of this study, and that of Essani (1983), ruled out the appearance of IgE-selective suppressor cells or IgE-suppressive factors. Furthermore, evidence for the production of regulatory auto-anti-idiotypic antibodies was not demonstrable. In regard to a possible mechanism operative through antibody feedback regulation, observations during the course of this study (not shown), and those of Ishizaka and Okudaira (1972), indicated that ongoing IgE responses were not susceptible to suppression by passively administered antibody. However, those studies did not examine the possibility that IgG isotypes may differentially regulate ongoing IgE production. It was possible that anti-DNP IgG2a was much more effective than IgG1 in regulating IgE synthesis. The results of the adoptive transfer of hybridomas producing either IgG1 or IgG2a into TNP-KLH-pretreated mice demonstrated however that this was not the case (Table 22A). Although the possibility exists that the levels of anti-DNP IgG were not high enough in the recipient mice to exert a suppressive effect, the levels were nonetheless comparable to those found in mice pretreated with DNP-BP at the time of immunization with DNP-OA (Figures 10,11; Table 22B).

We propose therefore, that the most likely explanation for the modulation of the anti-DNP IgE response described in this study is that a large segment of the anti-DNP B cell

repertoire may have been induced to express and secrete IgG2a antibodies under the influence of T_H1, the predominant T helper cell subset, generated by DNP-BP. Once committed to IgG2a, it is unlikely that these cells could switch to other isotypes (Lutzker et al., 1988; Stevens et al., 1988). Immunization with DNP-OA in alum, would activate OA-specific T_H cells in which the T_H2 subset predominates. T_H2 cells, in turn would collaborate with the limiting number of DNP-specific IgE B-cell precursors induced by the DNP-BP pretreatment. As no new DNP-specific IgE B cells can be recruited, the anti-DNP IgE response rapidly disappears after the day 7 peak.

According to this proposed mechanism, anti-OA IgE responses should remain unaffected, as they are induced by T_H2. Indeed, anti-OA PCA titers were the same, regardless of whether the mice were pretreated with DNP-BP or not (Table 18). Moreover the IgG1/IgG2a isotype distribution of the anti-OA response was also unaffected by pretreatment (Table 18). Further support for the proposed mechanism includes the finding that the administration of 10^8 DNP-BP to mice already immunized with DNP-OA was without effect on the anti-DNP IgG isotype profile (Table 19). This would be expected if the T_H cell repertoire had already been established as a result of immunization with DNP-OA. This rationale may also explain the uniqueness of the DNP-BP-pretreated environment (Table 3). Normal cells placed into a DNP-BP-pretreated recipient would be influenced by a

predominant population of T_H1 cells, whereas cells removed from a DNP-BP-pretreated environment would be influenced by the helper T cell population induced by the immunization of the recipient (DNP-OA in alum). Collectively, these results suggest that preferential induction of various T helper cell subsets through immunization might be responsible for the observed response.

The ability of a bacterial vaccine to stimulate IgG2a responses preferentially, and its failure to produce IgE was recently reported after the administration of Brucella abortus (Finkelman et al., 1987). This effect was attributed to the ability of the vaccine to stimulate IFN-gamma production. Treatment with anti-IFN-gamma antibody strongly suppressed IgG2a and stimulated IgG1, but not IgE production. It must be noted however that total isotype levels and not antigen-specific antibodies were measured in those studies.

Although several laboratories have already established a role for IFN-gamma in isotype-selective regulation, the majority of the investigations were carried out in vitro on either resting or polyclonally (LPS)-activated B cells (Coffman et al., 1988; Snapper et al., 1988). The results of this study are, to our knowledge, the first to demonstrate the control by IFN-gamma, of an antigen-specific immune response induced as a result of deliberate antigen administration, and the dependence of the isotype profile of this response on the nature of the antigen injected. One

must be cautious, however, when drawing a functional relationship between lymphokine production (detection) and its effects on an immune response, as the effects of any individual or group of lymphokines are known to be pleiotropic (O'Garra et al., 1988). Therefore, although the present study has implicated, and demonstrated, the roles of IFN-gamma in the modulation of an antigen-specific immune response, a more detailed examination of the effects of various helper T cell clones obtained from mice treated with DNP-BP/DNP-OA would be desirable.

Ideally, this analysis would take into account the clonal frequency of T_H1/T_H2 cells, as well as examine the effects of various antigenic stimuli on their lymphokine profiles. Monoclonal antibodies capable of discerning two distinct subsets of T_H cells in vivo have recently been described (Hayakawa and Hardy, 1988). It should be pointed out, that the contribution of IL-4 to the observed anti-DNP IgE response was not examined in this study. It is conceivable that the levels of IL-4 before and after immunization with DNP-BP and DNP-OA in alum may also dictate the kinetics and pattern of isotype expression. The relative influence of either IFN-gamma or IL-4 in vitro, or perhaps even in vivo, could be assessed by the administration of antibodies specific for each lymphokine.

Further analysis is also warranted in groups of mice immunized with BP alone, or BP together with hapten-carrier. It would be interesting to determine what makes the DNP-BP

conjugate unique, and whether the regulation of the response induced by DNP-BP/DNP-OA is mediated by cognate or bystander interactions between helper T cells and B cells.

The relevance of this mechanism for human disorders of the IgE system is difficult to assess but recent reports seem to suggest that similar, lymphokine-dependent regulatory mechanisms are involved (Del Prete et al., 1988; Pene et al., 1988). It is clear however, that a detailed analysis and understanding of the functional interactions of various T cell subsets and B cells after antigenic stimulation would greatly enhance our understanding of the immunoregulation of IgE-mediated allergic disorders.

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Appendix 1.

Statistical analysis of Results.

i) Anti-DNP responses of 9 individual mice immunized with 2μg DNP-OA in alum 14 days prior to bleeding.

IgE (PCA titers) titer	log_{10}	IgG (RIA) μg/ml	log_{10}
800	2.90	134	2.13
800	2.90	500	2.70
640	2.81	215	2.33
80	1.90	235	2.37
800	2.90	270	2.43
1600	3.20	300	2.48
800	2.90	249	2.40
160	2.20	150	2.18
1600	3.20	287	2.46

IgE log_{10}:

Minimum:	1.90	
Maximum:	3.20	
Range:	1.30	
Median:	2.90	
Mean:	2.768	(586)*
Standard error:	0.145	
Variance:	0.190	
Standard deviation:	0.436	

IgG μg/ml log_{10}:

Minimum:	2.13	
Maximum:	2.70	
Range:	0.57	
Median:	2.40	
Mean:	2.387	(243.8)*
Standard error:	0.056	

* = retransformed data values

ii) Comparison of the PCA titers of individual mice pretreated with 10^9 DNP-BP 14 days prior to immunization with 2μg DNP-OA in alum. The difference between the means of mice bled on day 7 and on day 14 relative to DNP-OA in alum was compared using the Student's t distribution test.

Day	7	14
Individual titers (PCA titer - $\log_{10}$)	320 - 2.51	20 - 1.3
	320 - 2.51	10 - 1.0
	160 - 2.20	40 - 1.6
	320 - 2.51	20 - 1.3
	160 - 2.20	20 - 1.3
$\log_{10}$ Minimum:	2.20	1.00
Maximum:	2.51	1.60
Range:	0.31	0.60
Median:	2.51	1.30
Mean:	2.386	1.300
Standard error:	0.076	0.095
Variance:	0.029	0.045
Standard deviation:	0.170	0.212

t-statistic: 8.937
Degrees of freedom: 8
Significance: 0.000 ($P < 0.005$)

Therefore, it is concluded that the mean titer value differences obtained between individual mice bled on day 7 and at day 14 after pretreatment and priming are significanly different.

iii) Single factor analysis of variance to determine if the treatment of mice with DNP-BP or TNP-KLH affected DNP-specific IgE PCA titers. 14 days prior to immunization with 2μg DNP-OA in alum, mice were pretreated as outlined in the table below. IgE titers were obtained from mice bled 14 days after immunization with DNP-OA in alum.

PCA titers - $\log_{10}$ transformed PCA titers.

DNP-BP-pretreated	No pretreatment	TNP-KLH-pretreated
20 - 1.30	320 - 2.51	640 - 2.81
10 - 1.00	160 - 2.20	640 - 2.81
20 - 1.30	640 - 2.81	320 - 2.51
10 - 1.00	160 - 2.20	160 - 2.20
40 - 1.60	320 - 2.51	320 - 2.51

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares
Total	6.161	14	
Groups	5.388	2	2.694
Error	0.773	12	0.064
F-Ratio	41.823		
Probability >	0.000		

Therefore, the probability that the variations in the anti-DNP IgE PCA titers observed on day 14 is not the result of pretreatment, is much less than 0.005%.