HAPTEN-SPECIFIC IMMUNOSUPPRESSION TRANSFERABLE BY SPLEEN CELLS OF MICE TOLERIZED WITH A CONJUGATE OF HYDROXY-IODO-NITROPHENYL ACETYL AND POLYVINYL ALCOHOL

BY

K. STEPHAN KRUEGER

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

Department of Immunology
University of Manitoba
Winnipeg, Manitoba

July, 1988

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ISBN 0-315-47991-4

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DEDICATION

"Attempt the end, and never stand to doubt;

Nothing's so hard but search will find it out."

Richard Lovelace (Seek and Find)

I dedicate this thesis to Verena, Simon and Nicholas.

Acknowledgements

I express my gratitude to:

Dr. A.H. Sehon for providing the original impetus, as well as superb encouragement over the entire span of the project; I also appreciated his constructive editorial assistance for the thesis;

Dr. W.Y. Lee for his guidance in the initial stages;

Dr. R.J. Schwenk for giving me a model for scientific self-criticism; he was also helpful in the proof-reading of my thesis;

Dr. V. Holford-Strevens for her invaluable comments on the thesis;

My student colleagues over the years for their steady reassurance;

The research assistants who helped me to establish some methodology, especially Valerie Cripps, Dr. Glen Lang, Motoko Miyamoto and Bill Stefura;

Dr. C.-J. Jackson for the method of calculating molar ratios of NIP/PVA from NMR spectra, which were determined in the Dept. of Chemistry by Mr. Kirk Marat.

I am eternally grateful to Verena for her patience and encouragement during the critical stage of this research project.

I wish to thank the Medical Research Council of Canada for providing me the opportunity -through grants in support of the MRC Group for Allergy Research- to participate in the challenging and fascinating process of scientific research, and I hope to repay the debt to the public trust in the future.

List of Abbreviations

1°	primary
2°	secondary
4B	Sepharose 4B agarose gel
λ	class of Ig light chain
μ	heavy chain of IgM
Ab	antibody
ABA	azobenzenearsonate
Ag	antigen
ACA-44	Ultrogel of polyacrylamide/agarose
$Al(OH)_3$	aluminum hydroxide adjuvant
APC	antigen presenting cell
B cell	bone marrow-derived lymphocyte
B6anti-NP	polyclonal anti-NP antibodies from C57BL/6 mice
B6NMS	normal mouse serum of C57BL/6 mice
B6nIg	normal immunoglobulins of C57BL/6 mice
BDF_1	hybrid mouse strain of C57BL/6 X DBA/2 ($B6D2F_1$)
B_ϵ	B cell producing IgE
BI	bovine insulin
BPO	benzylpenicilloyl group
BSA	bovine serum albumin
C′	complement
$CB6F_1$	hybrid mouse strain of BALB/c X C57BL/6
CH	methine
CH_2	methylene
$CrCl_3$	chromium chloride
CS	contact sensitivity

Cy	cyclophosphamide
c.p.m.	counts per minute
DCC	dicyclohexyl carbodiimide
ddH_2O	double distilled water
DNFB	dinitrofluorobenzene
DNP	dinitrophenyl residue
DNP-SC	conjugate of DNP with spleen cells
DTH	delayed type hypersensitivity
EtOH	ethanol
Fc	Fc fragment of an immunoglobulin
FCA	Freund's complete adjuvant
FCS	fetal calf serum (or fetal bovine serum)
FcR	receptor for Fc
$Fc_{\epsilon}R$	Fc receptor specific for IgE
FITC	fluorescein isothiocyanate
Flu	fluorescein
F/T_E	freeze/thaw extract of cells
GAT	random copolymer of Glu, Ala, Tyr i.e. (Glu^{60}, Ala^{30}, Tyr^{10})
GEF	glycosylation enhancing factor
GIF	glycosylation inhibiting factor
GT	random copolymer of Glu and Tyr i.e. (Glu^{50}, Tyr^{50})
G-25, G-75	cross-linked dextran gels also referred to as Sephadex
HCl	hydrochloride
HGG	human gamma globulin
H-2	histocompatibility-2 region of the MHC
id	idiotype

IgE	immunoglobulin E (epsilon heavy chain)
IgG	immunoglobulin G (gamma heavy chain)
Igh-V	variable region of the heavy chain gene
IgM	immunoglobulin M (mu heavy chain)
I-A	portion of H-2 involved in antigen presentation
I-J	considered formerly as portion of H-2 linked to suppression (actual location unknown)
i.p.	intraperitoneal
i.v.	intravenous
kD	kilo Daltons
Lyt1, Lyt2	lymphocyte surface markers
mAb	monoclonal antibody
MHC	major histocompatibility complex
Mϕ	macrophage
mRNA	messenger ribonucleic acid
M.W.	molecular weight
$NaHCO_3$	sodium bicarbonate
NaN_3	sodium azide
Nb	*Nippostrongylus brasiliensis*
NGS	normal goat serum
NH_4Cl	ammonium chloride
NHS	N-hydroxy succinimide
NIP	4-hydroxy-3-iodo-5-nitrophenyl acetyl residue
NIP-*O*-succ	NIP-*O*-succinimide
nl	normal or control mice
NMIg	normal mouse immunoglobulin
NMR	nuclear magnetic resonance

NMS	normal mouse serum
NNP	4-hydroxy-3,5-dinitrophenyl acetyl residue
NP	4-hydroxy-3-nitrophenyl acetyl residue
NW T	nylon wool-purified T cells
OA	ovalbumin
O.D.	optical density
OH	hydroxyl
PBS	phosphate-buffered saline, pH 7.4
PCA	passive cutaneous anaphylaxis
PEC	peritoneal exudate cells
PEG	polyethylene glycol
PFC	plaque-forming cell
PVA	polyvinyl alcohol
$PVA\text{-}NH_2$	amino-derivatized PVA
P.F.	potentiating factor
RaMIg	rabbit anti-mouse immunoglobulin
RIA	radioimmunoassay
r.t.	room temperature
SAC	splenic adherent cells
SAS	saturated ammonium sulphate
SC	whole spleen cells
SC_E	extract of spleen cells
Seph 4B	Sepharose 4B agarose gel
ShaMIg	sheep anti-mouse immunoglobulin
sIg	surface immunoglobulin
SRBC	sheep red blood cells
s.c.	subcutaneous

S.F.	suppressive factor
T cell	thymus-derived lymphocyte
T_{DH}	T cell responsible for delayed hypersensitivity
T_E	extract of sonicated T cells
T_h	helper T cell
ThF	T helper factor
TM	trimellityl residue
TMA	trimellitic anhydride
T_N	T cells from normal or control mice
tol	mice tolerized by hapten-PVA treatment
TNP	trinitrophenyl
T_s	suppressor T cell
Ts_1, Ts_2, Ts_3	subpopulations of T cells in suppressor circuit
TsF	T suppressor factor
T_T	T cells from mice treated with hapten-PVA conjugate
V_H	variable region of Ig heavy chain genes

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ABSTRACT

Injection of various strains of mice with 1-2 milligrams of NIP_4-PVA prior to immunization with NP_4-OA or NIP_4-OA markedly suppressed (>90%) the ability of these animals to develop a primary anti-NIP IgE response. Transfer of spleen T cells from NIP-PVA treated donor C57BL/6 mice or the F/T extracts of these cells to naive isologous mice also led to suppression of NIP-specific IgE and IgG responses in the recipients. However, on further identical transfers, nanogram amounts of NIP-PVA were found associated with $2x10^7$ spleen T cells (30-190 ng) and with their corresponding F/T extracts (10-75 ng). Similar nanogram amounts of NIP-PVA could be pulsed *in vitro* onto $2x10^7$ spleen T cells from naive mice, and the F/T extracts of these cells could also suppress IgE responses in recipient mice. Since extracts of normal spleen T cells pulsed with DNP-PVA did not suppress anti-NIP IgE responses, it was concluded that only conjugates of PVA with the appropriate hapten (NIP) could confer on the T cell extracts the ability to suppress anti-NIP IgE responses.

At this point, it was necessary to determine whether or not the suppressive effect by 10-75 nanograms of NIP-PVA required tight association of NIP-PVA with cellular components of normal spleen T cells. Compared to NIP-PVA present in spleen T cell extracts, soluble NIP-PVA by itself had the same properties with respect to the molecular weight estimated by ultrafiltration, binding to NIP-specific antibody and its suppressogenic effectiveness at nanogram doses. Therefore, these results were considered to indicate that free NIP-PVA at less than 100 ng could mimic the effects of the

suppressive cell extracts containing NIP-PVA. Furthermore, the suppression induced by free NIP-PVA in nanogram doses was abrogated by cyclophosphamide, which is known to particularly affect suppressor cell populations, whereas the suppression by one milligram of NIP-PVA was unaffected by cyclophosphamide.

From all these results it was concluded that nanogram doses of NIP-PVA associated with spleen T cells of NIP-PVA treated mice (i.e. carried-over NIP-PVA) could account for the transfer of suppression by these T cells. However, on the basis of the observation that cyclophosphamide reversed the suppression, the possibility that nanogram doses of NIP-PVA may preferentially induce an indirect form of tolerance mediated by suppressor cells could not be ruled out. In contrast, it appeared that milligram doses of NIP-PVA directly rendered B cells tolerant, since this form of tolerance was not abrogated by cyclophosphamide. In support of this conclusion, various selective methods had failed to identify suppressor T cells in the spleens of mice tolerized with milligram doses of NIP-PVA.

Introduction and Rationale

The main purpose of this study was to further elucidate the mechanisms involved in the suppression of antibody production by conjugates of hapten with polyvinyl alcohol (PVA). A conjugate consisting of the hapten 4-hydroxy-3-iodo-5-nitrophenyl acetyl (NIP) coupled to a non-immunogenic carrier backbone of PVA was chosen for the investigation of suppression of NIP-specific IgE as well as IgG responses.

The PVA polymer, consisting of (CH_2CHOH) units, is hydrophilic and non-immunogenic (60). From previous studies in this laboratory (reviewed in 61) it was concluded that conjugates of PVA with certain haptens (i.e. DNP and TM) tolerized hapten-specific B cells when administered i.p. in a dose of 1 mg one day before immunization of mice with the appropriate hapten-OA conjugate. The mechanism for this type of direct tolerance may be envisaged as being due to the binding of polyvalent hapten-PVA to hapten-specific B cells resulting in receptor blockade and in the consequent inactivation or elimination of these cells (99). The blockade effect is long-term and even the radiation-resistant component of the IgE response can be suppressed for up to 6 weeks (47).

The indirect suppressive effects of hapten-PVA conjugates involving activation of DNP-specific suppressor T cells have also been documented (61), viz., injections of DNP-PVA conjugates with an average epitope density of 1.4 was reported to elicit T_s cells, which could downregulate the DNP-specific IgE response upon transfer to naive recipients. In the early experiments of the present study, with DNP and Flu as haptens, it was confirmed that transfer of suppression could be achieved with spleen cells harvested from donor mice between

5 and 22 days after hapten-PVA was injected. The studies described in this thesis were initiated by a desire to produce suppressor factors from suppressor cells induced by i.p. injections of NIP-PVA into mice.

The choice of NIP as the haptenic group and of C57BL/6 mice for this study was based on the fact that the primary antibody response to NP in this strain consists predominantly of antibodies carrying λ-light chains and expressing a major idiotypic marker, namely the NP^b idiotype (50). Moreover, the affinity of antibodies bearing this id is higher for derivatives of NP (such as NIP and NNP) than for the homologous NP group. This property is termed heteroclitic fine specificity (68) and may be due to a tighter binding of anti-NP antibodies to a chemical state of NP (the phenoxide vs. the phenolic form at position 4 of the ring) which is reinforced by electron-withdrawing substituents (iodo or nitro) attached ortho to the 4-hydroxy component (68).

In another study in this MRC Group, it was shown that the fine specificity of NP-specific IgE antibodies resembled that of NP-specific IgM and IgG antibodies in the same mouse strain, $CB6F_1$ (69). Therefore, it was anticipated that NIP-PVA might interact with suppressor T cells exhibiting the predominant NP^b idiotype, with a higher affinity for the NIP than for the NP hapten. This anticipation was further supported by the results of Dorf and co-workers, who had demonstrated a sequence of suppressor T cells in C57BL/6 mice specific either for NP (NP^b id+) or specific for the NP^b id (id-) (62), i.e. Ts_1 (id+), Ts_2 (id-) and Ts_3 (id+). Hence, it was reasonable to hypothesize that injection of NIP-PVA might also evoke a suppressor cell cascade.

LITERATURE REVIEW

IgE-mediated allergic reactions

The term "allergy" was first coined by von Pirquet (110) to mean "altered reactivity" of the host, with no distinction made between beneficial and harmful responses. According to Bellanti (p. 14 in ref. 1) "immunity has come to mean that which von Pirquet defined originally as allergy, and allergy has come to mean hypersensitivity." Therefore, allergy represents "an altered reactivity to an antigen that can result in pathological reactions upon the exposure of the sensitized host to the particular antigen" (p. 292 in ref. 1). Currently 4 types of such hypersensitivity are recognized according to the factors involved, i.e. 1) cytotropic antibody leading to immediate hypersensitivity, 2) cytotoxic antibody leading to cell death, 3) antigen-antibody complexes leading to local or systemic inflammation and 4) cell-mediated delayed hypersensitivity leading to local inflammation. For the purpose of this review, only hypersensitivities of the types 1 and 4 will be described.

In the type 1 reaction homocytotropic IgE (and IgG) antibodies are produced which can sensitize mast cells and basophils to release various vasoactive amines on re-exposure to allergen. The events involving the mast cell as well as the effects of the mediators on the target cells occur rapidly -- hence the term immediate hypersensitivity. Briefly, the $Fc_{\epsilon}R$ on the mast cell binds tenaciously the IgE molecules, and cross-linking of at least 2 cell-bound IgE's by allergen (natural conditions) or by anti-IgE (experimental conditions) activates a series of events both at and within the cell membrane (30, 33). These events lead to degranulation and release of hista-

mine, leukotrienes, serotonin and/or other vasoactive mediators, which are responsible for the symptomatic manifestations of the allergic state in man.

The type 4 reaction, also referred to as DTH, is an inflammatory reaction due to infiltration of specific T cells and phagocytes (77). One experimental model for DTH in the mouse is contact sensitivity (CS) which has been described in detail by Mekori and Claman (78). Briefly, CS is readily induced by epicutaneous application of the sensitizer to the intact skin, a process which imitates the clinical situation in humans. Elicitation of the CS is achieved by epicutaneous application of the sensitizer or cross-reacting derivatives, with the maximum reaction observed 24-48 hrs later. The key point of this system is that T cells alone can transfer the state of DTH, and the subpopulation of T cells responsible for CS are referred to as T_{DH}.

A role for IgE in the CS response has been postulated by various authors, in particular Ray *et al.* (89). These authors reported that DNP-specific monoclonal IgE Ab's could presensitize mice to produce a biphasic pattern of ear swelling upon challenge with dinitrofluorobenzene. Furthermore, Kennedy (90) recently communicated that skin sensitization with picryl chloride, FITC and NP-*O*-succinimide induced IgE antibody responses specific for the haptens TNP, Flu and NP, respectively.

Regulation of IgE production

Pharmacological treatment of allergies resulting from elevated IgE levels has focussed primarily on the blockade of components involved in mast cell and basophil degranulation, and on the inhibition of the effects of their secreted products, such as histamine. Drugs which perform these tasks are purely palliative, as they do not affect the levels of IgE, which is responsible for manifestations of hypersensitivity. Furthermore, because of the side-effects of these drugs, it would be desirable to develop a long-term cure for allergies, resting on the control of IgE antibody production.

Another therapeutic intervention, i.e. hyposensitization therapy, involves a series of injections of the offending allergens over the period of years with as yet undefined mechanisms. However, the rationale for this therapy is to increase the level of IgG antibodies, which may lead to a blocking effect, i.e. by competition with IgE antibodies for determinants on the allergen (98). Treatment of allergic patients by hyposensitization has resulted at times in anaphylactic shock, putting the patient's life in danger. Clearly, a more beneficial and truly curative approach would be to suppress the production of specific cytotropic antibodies.

Different model systems have been employed to manipulate the IgE response [reviewed by Tada (35) and Katz (34)]. It is pertinent to note that in mice, IgE Ab responses are regulated by genes in the H-2 region of the MHC. Levine and Vaz (2) observed that the production of persistent high titers of IgE Ab in the mouse required one to administer *extremely low doses* of antigen (1 μg or less) with aluminum hydroxide gel as adjuvant. Utilizing this model, several authors

succeeded in suppressing hapten- or antigen-specific allergic responses by prior injection of modified allergens, or of allergens and haptens coupled to non-immunogenic carriers (36-43). These studies have indicated two possible mechanisms of tolerance, one involving direct tolerance of the B_ϵ cell and the other indirect tolerance via the effect of suppressor T cells on B cells or on other T lymphocytes. These two mechanisms of tolerance are discussed below.

<u>Induction of tolerance with cell membrane-coupled haptens or antigens.</u>

Battisto and Bloom (11) first reported the i.v. injection of membrane-coupled hapten (DNP coupled to spleen cells) as a means of inducing tolerance to the hapten in the DTH response. This work prompted further studies by Claman and co-workers on the nature of tolerance induction by membrane-associated DNP (12). They used various hapten-modified cells to induce tolerance, including erythrocytes (RBC), spleen, lymph node, thymus and peritoneal exudate cells (PEC). The authors showed that DNFB coupled to syngeneic erythrocytes (DNP-RBC) efficiently induced tolerance to DNP, although the effect was slower in comparison to the rapid induction of tolerance by DNP-coupled spleen cells. In a subsequent paper, Miller *et al.* (17) determined that two types of tolerance were operating after i.v. injection of hapten-modified self-membrane (DNP-SC). Direct inhibition of DNFB-reactive T cell clones was initiated within one day, while the activation of T_s cells required one week before transfer of the T cells to recipients and could be prevented by treatment with cyclophosphamide (Cy). The treatment with Cy did not affect the

"phenotypic tolerance" (tolerance in donor mice).

In a further investigation of the cellular requirement for rapid induction of tolerance (or clonal inhibition) several factors were operational: the time between injection of the tolerogenic form of the hapten and immunization, as well as the tissue source of the cell used as the carrier and the number of cells injected (24). It was concluded that the most efficient cells for direct tolerance induction were haptenated B cells and/or splenic adherent cells. The degree of tolerogenicity for different populations of cells was as follows: spleen> lymph node> CRT (cortisone-resistant thymocytes) and thymocytes, where the abundance of Ia+ cells was as follows: 52>35>10%. Hence, cells rich in Ia expression were also more efficient at inducing direct tolerance. The cells also had to be intact, but not viable, since heat killing did not affect the ability of the cells to induce tolerance.

Direct B cell tolerance (rather than T cell tolerance as above) was induced by Scott (15) with haptenated moieties shed from TNP-coupled splenocytes. The *in vitro* Ab response to TNP could be inhibited by TNP-SC, even if the responding spleen cells were T-cell depleted. This implied that T cells were not required as intermediaries for this tolerance induction. Direct tolerance could also be achieved by hapten coupled to teratoma cells, which lacked serologically detectable H-2. This agreed with the findings of Miller *et al.* (16) and Long (53) that haptenated allogeneic cells were also tolerogenic. On the other hand, the induction of suppressor T cells (an indirect form of tolerance) was more stringent, requiring some H-2 compatibility [such as in the H-2 K and H-2 D re-

gions; Miller *et al.* (16)]. The generation of T_s by DNP-LC was restricted by the H-2 region, but the subsequent activity was not genetically restricted; furthermore, these T_s acted at the efferent stage of the response. To further characterize the membrane determinants reponsible for inducing tolerance, cell lysates were prepared and passed over various immunosorbents. For both direct and indirect types of tolerance, DNP modification of the self-membrane was required. However, direct (phenotypic) tolerance could be achieved without associated H-2 determinants, whereas the induction of T_s required H-2 D or H-2 K determinants on the haptenated cell lysates. The induction of suppressor T cells in other systems is also MHC-restricted, namely for *in vitro* Ab responses to erythrocytes (18) and haptens (19) and for cell-mediated immunity to viruses (20), haptens (21) and allogeneic cells (22,23).

Recently, Yokomuro *et al.* (26,27) have used cells which were only briefly pulsed instead of covalently coupled with antigen to induce suppression of the IgE response. The rationale for using the pulsing procedure was that normally spleen cells *in situ* would probably take up antigens *in vivo* in the same way as spleen cells pulsed with antigens *in vitro*. This procedure for joining antigen to syngeneic spleen cells is more physiologically relevant than chemical coupling, which is normally used for haptenating spleen cells. They found that the route of administration of the antigen-pulsed syngeneic spleen cells determined the effect. The order of direct tolerogenicity was intravenous> intraperitoneal> subcutaneous. This was in agreement with the original finding of Greene *et al.* (82) that TNP-coupled syngeneic cells induced suppression of CS when administered i.v.,

while the same cells induced CS when injected subcutaneously. In Yokomuro's system, the major target of an immediate tolerance was the carrier-specific T cell. Furthermore, antigen-pulsed T cells were best at inducing this type of immediate tolerance. Yokomuro and co-workers also induced suppressor T cells detectable by day 7 following the i.v. injection of either antigen-pulsed adherent or non-adherent subpopulations of spleen.

In the system of Sherr *et al.* (54), in contrast, it was found that only adherent cells coupled with 100 ng palmitoyl fowl gamma globulin (p-FGG) were capable of inducing a transferable state of non-responsiveness due to suppressor cells for IgM and IgG responses. Furthermore, as low as 10^3 p-FGG-coupled unfractionated spleen cells were sufficient to induce this suppression. Similarly, Usui *et al.* (25) found that NP-adherent cells were at least 1000-fold more efficient at inducing Ts_1 activity in the CS response to NP than NP-coupled nonadherent cells.

Recent work by P. Maiti in this laboratory indicated that BI-coupled adherent cells (either from PEC or from a suspension of P388D1 cells in culture), injected into mice before immunization with BI in Freund's complete adjuvant, could induce suppressor T cells specific for BI (56). Previously, Kudo *et al.* (86), also in this laboratory, had shown that the IgE response of BDF_1 mice to BI could be suppressed by the i.v. injection of BI-coupled syngeneic spleen cell conjugates; this finding was confirmed with other mouse strains as well (87).

Suppressor cells and suppressor factors specific for antigen

In order to achieve long-term suppression of an antibody response, it would be desirable to engage the suppressor T cells specific for the hapten or antigen in question. Ever since the original report of Gershon and Kondo (4), describing antigen-specific suppressor T cells following antigen administration, suppressor T cells have preoccupied many workers involved in the study of immune tolerance. The mechanism underlying allergy might be the breakdown of tolerance or a hyper-responsiveness to foreign substances, leading to IgE production. The identification of antigen- or hapten-specific suppressor T cells provided the impetus for the modification of overt immune responses by activation of these T cells.

Pierce and Kapp (13) and Sy *et al.* (14) demonstrated that the spleen was the major organ of suppressor cell accumulation. However, Asherson *et al.* (52) showed that mice splenectomized 13 days before tolerization with picryl sulfonic acid still developed suppressor T cells in their lymph nodes. In a later paper, Brideau *et al.* (29) reported more suppressor cell precursors in the spleen than in the lymph node.

An alternative approach to the injection of tolerogenic forms of antigen for inducing suppressor cells was to inject anti-idiotypic antibodies to select for idiotype-positive suppressor cells. Malley (88) described the use of anti-idiotypic antibodies for suppressing IgE responses to timothy grass pollen. The anti-idiotypes were produced either against ThF from cultured T cells of antigen-primed mice or against the IgE's of hyperimmune mice. A dose of 1-10 μg of either anti-idiotype could induce suppressor T cells in the spleen of

mice which downregulated a secondary timothy IgE response in subsequent recipients. Further work from the same laboratory suggested that the induction of suppressor cells by anti-idiotype was dependent on an FcR-bearing "presenting" cell, since the Fc portion of the anti-idiotype was critical.

By employing anti-μ treated mice, HayGlass *et al.* (28) were able to show that the idiotypic repertoire of the suppressor cells in the ABA system depended on the exposure of precursor cells to id+ Ab in the circulation. These results generally support the hypothesis that Jerne's idiotype network interactions are involved in the generation of effector suppressor cells.

Suppressor factors derived from suppressor T cells have been analyzed by various laboratories [reviewed earlier by Altman and Katz (5) and Germain and Benacerraf (6)]. The first studies on suppressor factors from thymocytes or splenocytes were performed by Tada and colleagues, who showed that the sonication of Ascaris-specific suppressor T cells yielded a functionally antigen-specific suppressor factor (7). Pierres *et al.* (10), reported a difference in mode of action between *in vivo* and *in vitro* derived TsF's specific for GAT. They commented that "suppressor factor preparations may contain several distinct materials", highlighting the difficulties inherent in crude cell extracts or supernatants. In the GAT or GT system of Theze *et al.* (8) specific suppressor extracts were obtained from both GAT non-responder strains, and GT suppressor-type strains. The I-J phenotype, initially associated with a locus in the H-2 complex, was ascribed to these suppressor factors since they bound to anti-I-J antibodies (108). Recently, B. Diamond, M.E. Dorf and co-workers (91)

have produced monoclonal anti-idiotypes to anti-I-J^k mAb WF8.C12.8, which bind to the combining site of WF8.C12.8 and imitate a portion of I-J^k. These anti-idiotypes could prevent NP-coupled splenic adherent cells from inducing T_s cells, presumably through the binding of the anti-idiotype to a molecule on the macrophage membrane essential for the induction of suppressor cells. These results further support a view of I-J molecules as recognition molecules for Class II MHC determinants (100).

The NP/NIP system with reference to immunoregulation.

The antibody response to the hapten NP has been particularly well-studied since 85% of primary NP-specific Ab's in C57BL/6 mice bear a predominant idiotype (NP^b), linked to the Igh-V gene of the *b* haplotype (50). Dorf and co-workers (32a) have used this NP^b system to extensively analyze the role of idiotypes in the regulation by suppressor T cells. They identified a suppressor cell cascade with three subsets of T_s cells, i.e. Ts_1, Ts_2 and Ts_3 cells, interconnected by their respective suppressor factors. These suppressor cells could be manifested in both DTH and PFC responses. MHC restrictions for suppressor activity are imposed by specialized populations of I-J bearing accessory cells, which present antigen or the suppressor factors. The Ts_1 is induced by NP-modified I-A or I-J bearing syngeneic adherent cells.

According to the same authors (3), the Ts_1 (inducer cell; id+, I-J+, Ly1+) releases TsF_1, which after binding to a factor presenting cell (adherent cell, FPC) stimulates a Ts_2 precursor (transducer cell; id-specific, I-J+, Lyt2+), which in turn produces a TsF_2 which

is presented by an FPC to a Ts_3 precursor (effector cell; id+, I-J+, Lyt2+). The Ts_3 precursors in naive spleens require prior stimulation by antigen on an I-J bearing APC as well as idiotype-specific B cells for differentiation to functional Ts_3 cells, capable of responding to TsF_2. The Ts_3 cell finally stimulates an effector cell to produce an antigen non-specific suppressor factor.

Suppressor factors specific for different determinants such as NP (32), ABA (9) and GAT (10) have been shown to bear idiotypic determinants that cross-react with the idiotypes on hapten-specific antibodies. In all systems, a predominant id has been used to identify different subsets of the suppressor cell cascade, as well as their TsF's, in terms of their bearing the idiotype (id+) or binding the id (id-).

The production of hybridomas for suppressor T cells, in combination with high pressure liquid chromatography separation, has greatly improved the characterization of suppressor factors (3, 3a). Immunochemical and biochemical analyses of suppressor factors have partially revealed their properties, i.e. an I-J bearing chain and an antigen-binding chain comprising a 2-chain factor in the SRBC and the ABA sytems (97), a 2-chain factor for effector TsF's from NP-specific hybridomas (106), a single chain TsF_1 and 2-chain TsF_2 for the GAT system (107) and a 2-chain TsF_1 in the GT system (112).

In summary, Asherson *et al.* (81) have tried to unify the work of various laboratories on suppressor cell circuits by suggesting that in most systems the sequence of suppressor cells begins with an inducer cell, which can stimulate a transducer cell leading to an effector cell or which can directly stimulate an effector cell.

IgE-binding factors and class-specific suppression.

Recently Ishizaka *et al.* (72) reviewed their studies of IgE-binding factors from rats infected with the nematode *Nippostrongylus brasiliensis* (Nb). Mesenteric lymph node (MLN) T cells isolated from these rats were shown to produce an IgE suppressive factor (S.F.) 8 days after infection, and an IgE potentiating factor (P.F.) 14 days after infection. The major structural difference between the P.F. and S.F. was that the former had both N-linked, mannose-rich oligosaccharides and O-linked oligosaccharides, while the latter had only O-linked oligosaccharides. By recombinant DNA methodology, it was also shown that the P.F. and S.F. share a common precursor, and that the nature of IgE-binding factors was determined by the post-translational glycosylation process.

To further elucidate the role of glycosylation, the Ishizaka group identified two T cell factors which either enhanced or inhibited glycosylation of IgE-binding factors obtained from mice and rats. Glycosylation enhancing factor (GEF) selectively enhanced the N-glycosylation of IgE-binding factors (the latter derived from Lyt1+ or W3/25+ T cells) during their biosynthesis. In the rat, antigen absorbed onto $Al(OH)_3$ induced not only T_H cells, but also a subset of T cells which released GEF upon antigenic stimulation. Pertussigen from Bordetella pertussis was also found to stimulate GEF formation from the same T cells. Alternatively, $Fc_{\epsilon}R+$ T cells selectively formed IgE suppressive factor when stimulated with IgE or interferon in the presence of glycosylation inhibiting factor (GIF). GIF was derived from Lyt2+ T cells obtained from mice treated with FCA. Antigen-specific suppressor T cells also were found to release

antigen-specific GIF.

In summary, then, GEF and GIF, acting on the same T cells capable of producing P.F. or S.F., determined the biological activity of the IgE binding factor after its translation from mRNA. GEF led to P.F., which enhanced the overall IgE response of rodents. GIF, on the other hand, engendered S.F. which could suppress the 1° IgE and IgG responses in mice, as well as the 2° IgE response. Interestingly, when repeated GIF treatment was given to mice undergoing a 1° response to DNP-OA both the IgE and IgG responses were suppressed, while the transfer of T cells from OA-primed, GIF-treated mice suppressed primarily the IgE response (79). Thus, in addition to antigen-specific suppression of both IgE and IgG, GIF could modulate $Fc_{\epsilon}R$+ T cells to form S.F., acting only on the IgE response.

The same authors observed that antigen-specific GIF resembled other antigen-specific suppressor factors, in that GIF obtained from antigen-specific suppressor cells had a M.W. of about 30 kD, expressed I-J determinants and had affinity for antigen.

In a system developed by Kishimoto *et al.* (75) DNP-Mycobacterium primed T cells could also produce IgE class-specific suppressor factors (IgE-TsF). The suppressive effect of these factors was antigen nonspecific, although DNP-specific stimulation was required for their induction. The target cells of this indirect suppression were found to be B_{ϵ} cells. As mentioned in the previous section, it is not unusual for the final effector suppressor factor to be antigen nonspecific.

Suppression of the IgE response with modified antigens or antigens coupled to non-immunogenic carriers.

Direct and/or indirect tolerance have been manifested in a number of different systems that have been used for the suppression of IgE, viz, 1) allergen-polysarcosine (poly-N-methylglycine) (36), 2) OA-pullulan (linear polymer of glucose) (37), 3) Protein-d-GL (co-polymer of D-glu and D-lys) (38, 39), 4) DNP-d-GL (40), 5) Hapten-CMC (carboxy-methyl cellulose) (41), 6) DNP-levan (a branched polyfructose) (43), 7) PEG-proteins (109,111) and 8) PVA-haptens (42). In system 1) a short-lived antigen-specific suppressor mechanism was evident. System 2) described an IgE-selective suppression which was transferable 2-4 wks after treatment. In 3) with the antigens OA and Antigen E of ragweed pollen, a long-lasting, Ag-specific, IgE-restricted suppression was attributed to helper T-cell tolerance, while for the timothy grass pollen antigen fragments, AgD_1 and AgD_2, Malley and co-workers found B cell blockade to be the mechanism of tolerance. System 4) involved B cell tolerance, due to lack of re-expression of functional receptors on the B cell. However, more recently, Kim *et al.* (44) reported that *in vitro* DNP-d-GL could induce hapten-specific suppressor cells cross-reactive with TNP on the same carrier, using a 10-fold higher dose than that used by Nossal *et al.* (45). The conjugate used in 5) inactivated B cells by clonal deletion, while in 6), B cell tolerance was operant. For 7) and 8), both mechanisms of suppression were shown to be operational depending on the nature of the conjugate used.

<u>The use of PVA as a non-immunogenic carrier</u>

Previous work (46, 42) has shown that conjugates of PVA with certain haptens, in particular DNP, BPO and TMA, directly tolerizes B cells with specificity for the appropriate hapten. Furthermore, allergic symptoms in mice have been abrogated by prior treatment with BPO-PVA and TM-PVA conjugates. The mechanism for this type of direct tolerance was postulated to involve receptor blockade, with the immunoglobulin receptor for hapten being prevented from interacting with subsequent immunogenic forms of the hapten. More recently, Wei *et al.* (99) showed by several criteria that B cells specific for the hapten TMA were irreversibly tolerized by TM-PVA treatment and that a central mechanism of B cell tolerance may be involved rather than a receptor blockade. The tolerogenic effect was found to last for at least 4 weeks and even persisted in the radiation-resistant component of an IgE response for up to 6 weeks (47).

Hapten-PVA conjugates also induced an indirect mode of suppression, which could be transferred by the spleen T cells of the treated donors. Lee and Sehon (48) identified DNP-specific suppressor T cells which were detectable 7 days after treatment of mice with DNP-PVA. Soluble extracts obtained from these T cells were also found to be suppressive (49), although the nature of the suppressive moieties was not determined. At the time this investigation was begun, the initial aim was to characterize hapten-specific T_s cells in an idiotypically well-defined system, namely the NP system (50).

MATERIALS AND METHODS

DNBS

2,4-dinitrobenzenesulfonic acid, sodium salt; M.W. 270.2, Lot no. 627145; ICN Pharmaceuticals, Inc., Plainview, NY.

$PVA_{14,000}$

$HO-[CH_2-CH(OH)-]_nCH_2CH_2OH$; 100% hydrolyzed, avge M.W.= 14,000; Aldrich Chemical Co. Inc.; Milwaukee, WI.

1,6-Hexanediamine

70% in water; $NH_2(CH_2)_6NH_2$; M.W. 116.21; Eastman Kodak Co., Rochester, NY.

5-Fluorescein Isothiocyanate

M.W. 389.38; Eastman Kodak Co., Rochester, NY.

Ficoll-metrizoate medium (density = 1.090) for separation of mouse splenocytes.

Solution A: Ficoll-14% solution

Ficoll-400 (Pharmacia, Montreal, PQ) was dissolved with stirring overnight in 100 ml ddH_2O.

Solution B: Na Metrizoate- 32.8% w/v (Nyegaard & Co. A/S, Oslo, Norway)

1. 30 ml of Solution B was added to 72 ml of Solution A.
2. 100 mg of NaN_3 was added to this mixture to prevent contamination, while stirring, and the resulting separation medium was stored in the dark at $4^{\circ}C$.

CNBr

M.W. 105.93; # 02080-25; Allied Fisher Scientific Co., Winnipeg, MB.

NIP-*O*-succinimide; M.W.= 420; Biosearch, San Rafael, CA.

NP-*O*-succinimide; M.W.= 294; Biosearch, San Rafael, CA.

Tris-NH_4Cl, pH 7.2

To 90 ml of 0.16M NH_4Cl was added 10 ml 0.17M Tris, followed by 1N HCl until the pH was 7.2.

1,4-Dioxane

M.W. 88.11; # D111-500; Allied Fisher Scientific Co., Winnipeg, MB. The dioxane was kept anhydrous with molecular sieves from Fisher, Grade 514, Type 4A (8-12 Mesh; Beads) # M-514.

Nylon wool

Fenwal code #4C2906; 35 gm pouch of scrubbed nylon fiber (3 denier, 3.81 cm, type 200); distributed by Baxter-Travenol, Deerfield, IL.

2% gentian violet

One gm gentian violet was added to 1% v/v glutaraldehyde in 50 ml and stirred overnight. The dye was filtered before use.

Phenylmethylsulfonyl fluoride (PMSF)

M.W. 174.2; Sigma Chem Co., Rochester, NY; No. P-7626

PMSF was dissolved in propan-2-ol, 43.5 mg/ml, with warming at 37°C for 1/2 hr. This solution could be stored for 1 wk. The final concentration in the medium for resuspending cells was 50 μg/ml.

Cyclophosphamide, monohydrate (Cy)

M.W. 279.1; Sigma Chemical Co.; No. C-0768

Cy was dissolved in PBS at 10 mg/ml and injected at 100 mg/kg i.p. into weighed mice.

Sheep anti-mouse Ig (developing antibody for PFC assay)

This antibody preparation was obtained by precipitation at 35% SAS from a sheep antiserum to normal mouse serum (a gift of Dr. K. Kelly, formerly of this laboratory). For the PFC assay, it was used at a 1:1000 dilution; 25 μl was added to the wells in which IgG antibody production was to be tested. It was shown to inhibit the number of IgM plaques by 46% on addition to AFC from mice undergoing a primary IgM response to SRBC. This was due most likely to anti-μ activity in the sheep anti-mouse Ig.

Rabbit anti-mouse Ig was donated by Dr. B.G. Carter

TM_{16}-BSA and TM_9-OA were prepared by Dr. Bing-Y. Wei of this laboratory, NP_{14}-BSA by M. Miyamoto, Fl_5-OA and Fl-goat gamma globulin by V. Cripps and DNP-NGS by K. Nawrocki.

$\underline{NIP_{36}\text{-HGG}}$, $\underline{NP_{36}\text{-HGG}}$, $\underline{NIP_{4}\text{-OA}}$, $\underline{NP_{4}\text{-OA}}$ or $\underline{NIP_{19}\text{-BSA}}$ were prepared by reacting 1 volume of NIP- or NP-*O*-succinimide in anhydrous dioxane with 5 volumes of the protein (20 mg/ml) in 0.2M $NaHCO_3$-Na_2CO_3, pH 9.0. After reaction at r.t. for 6 hrs and overnight at 4°C, the uncoupled NIP-*O*-succinimide was removed by dialysis against the same $NaHCO_3$-Na_2CO_3 buffer. The molar ratio of NIP-*O*-succinimide or NP-*O*-succinimide to protein required to achieve the above hapten densities was 45:1 for NIP_{36}-HGG and NP_{36}-HGG, 14:1 for NIP_4-OA and NP_4-OA, and 20:1 for the NIP_{19}-BSA conjugate.

The following extinction coefficients were used to determine the epitope density: ${}_{430}E^{M}$ (NIP)= $4.9x10^3$; ${}_{430}E^{M}$ (NP)= $4.23x10^3$; ${}_{280}E^{1\%}$ (HGG)= 14.3; ${}_{280}E^{1\%}$ (OA)= 7.35 and ${}_{280}E^{1\%}$ (BSA)= 6.67. Some absorption for NIP occurs at 280 nm as well as at the optimum of 430 nm, viz., for NIP on a PVA backbone, $O.D._{280}$= $0.96xO.D._{430}$ and for NIP conjugated to proteins the $O.D._{280}$= $0.68xO.D._{430}$. The contribution of NIP to the absorption at 280 nm must be taken into account when calculating PVA or protein concentrations from the $O.D._{280}$ values of the conjugate solutions.

$\underline{DNP_3\text{-OA}}$ was prepared by reacting 200 mg OA in 8 ml of 0.4 M Na_2CO_3 with 150 mg DNBS in 4 ml of 0.4M Na_2CO_3 and stirring at r.t. for 3 hrs. The free hapten was removed by gel filtration on a column (2.4 x 40 cm) of Sephadex G-25 equilibrated in PBS. The first yellow peak containing DNP-OA was collected. To determine the degree of substitution by DNP, the value of 17,400 for ${}_{360}E^{M}$ (DNP, ref. 114) was used; since DNP also absorbs at 280 nm (1/3 absorbance at 360 nm) its contribution must be subtracted from the total absorbance at 280 nm when determining the amount of OA.

Preparation of hapten-PVA conjugates

PVA_{14}-NH_2

$PVA_{14,000}$ (28 gms in 400 ml H_2O for $4.3x10^{-3}M$) was reacted with CNBr (10 gms for $2x10^{-1}M$). The pH was maintained at 10.5-11.0 with 2N NaOH. When the reaction was complete, 43.6 gm hexanediamine in 62.3 ml ($8.1x10^{-1}M$) was added and the mixture left at r.t. for 48 hrs.

The reaction mixture was dialyzed extensively against ddH_2O in a dialysis bag (Visking tubing) with a 12,000 Da cut-off, and lyophilized.

1. $DNP_{2.45}$-NH-PVA_{14}

To 400 mg PVA_{14}-NH_2 (batch= 31.08.78) in 20 ml ddH_2O (warmed to dissolve) was added 2 gm DNBS in 5 ml ddH_2O and 4 ml $1MK_2CO_3$. The mixture was stirred at r.t. overnight, the precipitate centrifuged out and the supernatant passed through G-25 in ddH_2O. The first coloured peak was dialyzed overnight in ddH_2O and freeze-dried.

2. $DNP_{1.45}$-NH-PVA_{14}

To 500 mg PVA_{14}-NH_2 (batch= 19.02.80) in 25 ml ddH_2O (warmed to dissolve) was added 2.5 gm DNBS in 12.5 ml ddH_2O and 5 ml $1MK_2CO_3$. The mixture was stirred at r.t. for 2 hr, the precipitate centrifuged out and the supernatant passed through G-25 in ddH_2O. The first coloured peak was dialyzed overnight in ddH_2O and freeze-dried.

3. $DNP_{1.4}$-NH-PVA_{14}

To 300 mg PVA_{14}-NH_2 in 15 ml ddH_2O (warmed to dissolve) was added 1.5 gm DNBS in 7.5 ml ddH_2O and 3 ml $1MK_2CO_3$. The mixture was

stirred at 37°C for 3.5 hr, and centrifuged at 4°C (10,000 r.p.m. for 10 min) before passage through a G-25 column 4x50 cm) in ddH_2O.4 The first coloured peak was dialyzed in ddH_2O and freeze-dried.

4. $\underline{Fl_{2.0}\text{-NH-PVA}_{14}}$

To 150 mg PVA_{14}-NH_2 (batch= 31.08.78) dissolved in 7.5 ml ddH_2O was added 15 mg FITC in 3.75 ml ddH_2O and 1.5 ml 1M K_2CO_3. The mixture was stirred at r.t. for 2 hr, the precipitate centrifuged out and the supernatant passed through G-25 in ddH_2O. The first coloured peak was dialyzed overnight in ddH_2O and freeze-dried.

5. $\underline{NIP_4\text{-NH-PVA}_{14}}$

To 430 mg PVA_{14}-NH_2 (batch= 11.05.81) dissolved with warming in ddH_2O (28 ml) was added 7 ml 1.0M $NaHCO_3$-Na_2CO_3, pH 9.0, and 145 mg NIP-*O*-succinimide in 7 ml anhydrous dioxane. NIP-*O*-succinimide was added in aliquots every 0.5 hr at r.t. over 6 hr and the mixture was left overnight at 4°C. After passage through a G-25 medium column of sufficient capacity (at least 5x volume of sample for desalting) the first coloured peak was dialyzed against ddH_2O twice and freeze-dried for analysis and use.

6. $\underline{NP_4\text{-NH-PVA}_{14}}$

The same method was employed as for 5., except that NIP-*O*-succinimide was substituted by an equimolar amount of NP-*O*-succinimide

Animals

Male 6-12 week-old BDF_1 mice and random-bred male Long-Evans Hooded rats were obtained from the Animal Care Facility of the University of Manitoba. Male 6-12 week-old C57BL/6 and BALB/c mice were purchased from Charles River, St. Constant, P.Q.. The recombinant mouse strains C3H/HeSnJ, C3H.SW/SnJ and B10.BR/SgSnJ were supplied by Jackson Laboratories, Bar Harbour , Maine. All animals were boarded in the Animal Care Facility of the University of Manitoba.

Calculation of the NIP epitope density of NIP_n-PVA conjugates

Analysis of NMR peaks (see Fig. 1)

		AREA (integration)			
^1H peaks:		methylene	methine[1]	OH's	NIP (aromatic)
Samples	mg	(CH_2)	(CH)		
PVA	7.26	36.61	16.59	8.20	
NIP-acetic acid	3.71				1.45, 1.46
PVA-NH_2	6.05	34.68	14.16	5.92	
PVA-NIP_4	4.55	24.85	10.55	4.84	.177, -[2]

[1] The methine peak was the most consistent relative to a given weight for each sample.

[2] The second aromatic peak overlapped with a third peak (possibly the phenolic OH) in the case of NIP attached to PVA; therefore, the integral of its area was indeterminable.

	AREA (integration)			
	mg	CH_2	CH	aromatic H
PVA standard	7.26	36.61	16.59	
PVA (integrals/mg)		5.04	2.29	
mg PVA in NIP-PVA		4.93	4.61	
PVA(-NH_2)	5.87	34.68	14.17	
PVA (integrals/mg)		5.9	2.41	
mg PVA in NIP-PVA		4.2	4.38	
NIP standard	3.71			1.45
NIP (integrals/mg)				.39
mg NIP in NIP-PVA				.45

Figure 1a. Proton (^{1}H)-NMR spectrum for PVA.

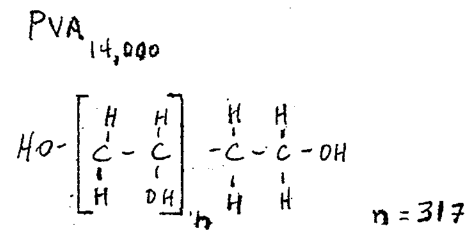
PVA, 1-H AT 300 MHZ, DMSOD6

PPM

PVA.501
DATE 23-10-85

SF	300.135
SY	112.350
O1	6500.000
SI	32768
TD	32768
SW	4000.000
HZ/PT	.244
PW	6.0
RD	4.000
AQ	4.096
RG	4
NS	128
TE	300
FW	5000
O2	3205.000
DP	60L DO
LB	.100
GB	.600
CX	37.00
CY	18.50
F1	10.122P
F2	.127P
HZ/CM	81.074
PPM/CM	.270
SR	4791.24

$PVA_{14,000}$



n = 317

NIP, 1-H AT 300 MHZ, DMSOD6

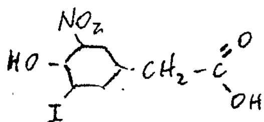
BRUKER

PPM

NIP.001
DATE 23-10-85

SF	300.135
SY	112.350
O1	6500.000
SI	32768
TD	32768
SW	4000.000
HZ/PT	.244
PW	6.0
RD	4.000
AQ	4.096
RG	4
NS	128
TE	300
FW	5000
O2	3205.000
DP	60L DO
LB	.100
GB	.600
CX	37.00
CY	18.50
F1	10.122P
F2	.127P
HZ/CM	81.074
PPM/CM	.270
SR	4791.24

NIP-acetic acid



3.6150
3.3658
2.5025
2.4964
2.4903
2.4842
2.4780

H_2O

aromatic H's

DMSO

$-CH_2-$

INTEGRAL

1.4517
1.4671
.0913
.1197
3.7163
.2090

integration high due to "tail" of H_2O peak

10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 .5

PPM

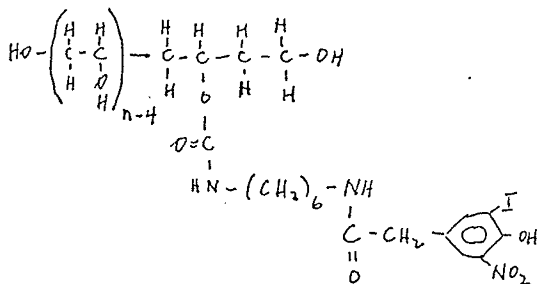
Figure 1b. Proton (^1H)-NMR spectrum for NIP-acetic acid.

PVA-NIP4. 1-H AT 300 MHZ. DMSOD6

PPM

PVANIP4.501
DATE 23-10-85

SF	300.135
SY	112.350
O1	6500.000
SI	32768
TD	32768
SW	4000.000
HZ/PT	.244
PW	6.0
RD	4.000
AQ	4.096
RG	4
NS	128
TE	300
FW	5000
O2	3205.000
DP	60L DO
LB	.100
GB	.600
CX	37.00
CY	18.50
F1	10.122P
F2	.127P
HZ/CM	81.074
PPM/CM	.270
SR	4791.24



3.8154
3.3636
2.5025
2.4964
2.4902
2.4841
2.4780
1.4314
1.4111
1.3812
1.3423

H2O
DMSO
OH's
CH2's
phenolic OH (probably)
aromatic H's
(-CH-) methine

INTEGRAL
.177
.361
.073
.100
4.038
10.550
24.846

10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 .5
PPM

Figure 1c. Proton (^{1}H)-NMR spectrum for $NIP_{4.1}$-PVA.

Peak signal	weight	% in NIP-PVA
CH_2	4.93	91.6
NIP	.45	8.4
total	5.38	100.0
CH	4.61	91.1
NIP	.45	8.9
total	5.06	100.0

M.W. PVA= 14,000

M.W. NIP-acetic acid= 323

PVA/NIP (M.W. ratio)= 43.3

(% weight) (M.W. ratio)

CH_2: NIP/PVA x PVA/NIP = 3.97

CH: NIP/PVA x PVA/NIP = 4.2

Average no. of NIP's/ PVA by the two methods of calculation, using either the methylene (CH_2) or the methine (CH) peaks of the PVA standard, is 4.1, which equals the epitope density calculated by absorption spectrophotometry of a solution of the same NIP-PVA.

From the above NMR profile it appeared that the OH groups on the hydrocarbon backbone of PVA were oriented mostly in the heterotactic form, with less in the iso- and syndio-tactic forms (95) indicating that there should be several neighbouring OH groups, required for reactivity with CNBr in the activation of PVA for eventual coupling to hexane-diamine.

Method for preparation of $N^{125}IP$-PVA.

	REAGENTS	AMOUNTS	moles	molar ratio
1.	Np-acetic acid	1 μg/ 100 μl	$5.0x10^{-9}$	3
	$Na^{125}I$	3 mCi/ 30 μl	$1.6x10^{-9}$	1
	Chloramine T	2.24 μg/ 100 μl	$8.0x10^{-9}$	5
	$Na_2S_2O_5$	2.13 μg/ 100 μl	$1.1x10^{-8}$	6.7

ORDER OF ADDITION

a) Np-acetic acid
b) $Na^{125}I$
c) PBS- 70 μl
d) Chloramine T- 25 μl at a time, every 15 min for 1 hr reaction

e) $Na_2S_2O_5$
f) PBS- 100 μl containing 250 μg of NIP-acetic acid as cold carrier

2. Preparation for TLC

a) The reaction mixture was acidified with 4 drops 1/3 N HCl (pH<2) in order to get below the pK_a of NIP-acetic acid (about 3.9).

b) The reaction mixture was extracted into $CHCl_3$ (2x0.5 ml) and dehydrated with $MgSO_4$, washing once with $CHCl_3$ (92% of total= $1.5x10^9$ c.p.m. in the organic phase)

c) The $CHCl_3$ was dried off to a volume of 100 μl or less.

d) The sample was spotted onto the TLC, in a line at the origin.

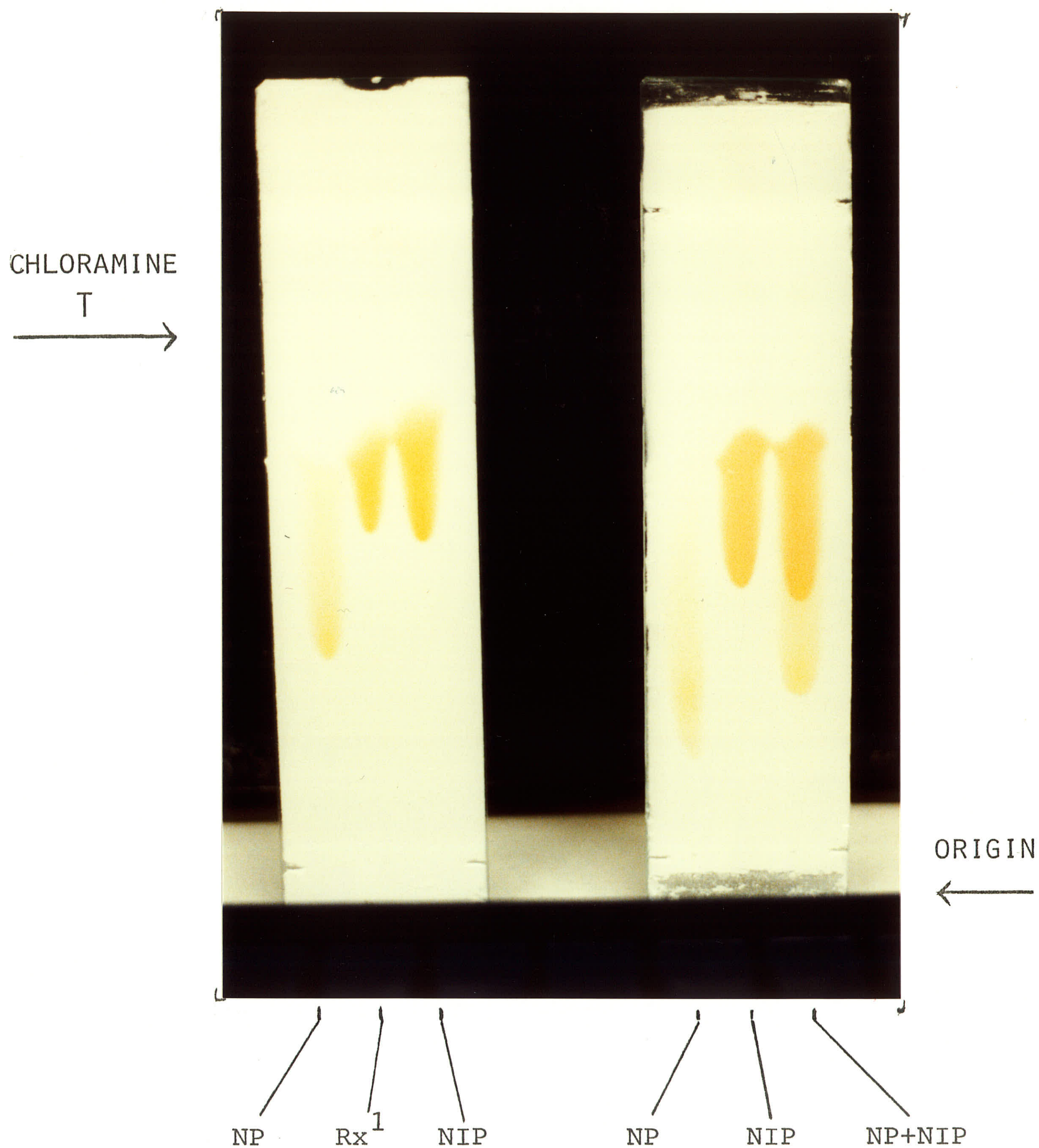
3. TLC separation

a) The TLC plate was run in the solvent 80:20 isopropanolol:25% NH_4OH.

b) The solvent was allowed to move to the top of the plate (see Fig. 2 for examples of the separation between NIP-acetic acid and NP-acetic acid).

c) The plate was dried and the silica containing the NIP-acetic acid was removed (mixture of hot and cold).

FIGURE 2. Thin-layer Chromatography Separation of NIP-acetic acid from NP-acetic acid.



[1]Conversion reaction of NP-acetic acid to NIP-acetic acid (see MATERIALS AND METHODS)

d) The sample was extracted with 10 % MeOH in $CHCl_3$ + acetic acid (1 drop/ ml solvent).

e) The solvent was dried off and the NIP-acetic acid resuspended in dioxane.

4. Conversion of NIP-acetic acid to NIP-*O*-succinimide

a) 0.25 ml dioxane containing 8 mg NIP-acetic acid (cold)

b) 1.0 ml dioxane containing $1x10^9$ c.p.m. $N^{125}IP$-acetic acid

c) 2.75 mg NHS in 0.1 ml dioxane

d) 5 mg DCC in 0.1 ml dioxane

e) Stirred 30 min at r.t.

f) Filtered through cotton plug.

Result: $7.0x10^8$ c.p.m. in 1.83 ml dioxane, in the form of 8.6 mg $N^{125}IP$-*O*-succinimide (solution A; specific activity = $8.1x10^7$ c.p.m./mg)

5. Preparation of $N^{125}IP$-PVA

a) To 39 mg PVA-NH_2 in 0.2 M carbonate-bicarbonate, pH 9.0, Solution A was added in 7 aliquots over 6 hrs at r.t., representing a molar ratio of 7.2:1 NIP-*O*-succinimide:PVA-NH_2.

b) The reaction was continued overnight at 4°C.

c) The first peak was isolated on a G-25 column (150 ml; 1.9cm x54 cm) using 0.2 M $NaHCO_3$-Na_2CO_3, pH 9.0 as the running buffer.

d) The sample was dialyzed 3 times against ddH_2O (10,000 x sample volume).

e) The sample was freeze-dried in a special apparatus for radioactive work and weighed out.

Result: 30.6 mg $(N^{125}IP)_3$-PVA; specific activity = $3.8x10^6$ c.p.m./ mg.

The conjugate $(N^{125}IP)_4$-PVA was similarly prepared, except for the following differences: the TLC chromatography in Section 3 was performed with the solvent 50% MeOH in ethyl acetate, in which only the NP-acetic acid moved with the solvent, while the NIP-acetic acid stayed at the baseline; the ratio of NIP-*O*-succinimide to PVA-NH_2 in Section 5 was 20:1; the specific activity of NIP-*O*-succinimide was $4.4x10^7$ c.p.m./mg and the final specific activity of $(N^{125}IP)_4$-PVA was $3.3x10^6$ c.p.m./mg.

Preparation of spleen cell suspensions

Mice were sacrificed by cervical dislocation. The spleens were removed and placed in MEM-HEPES before homogenization with a Kontes homogenizer that had been ground in order to be loosely-fitting. The resulting suspension was filtered through a coarse 40 mesh screen and a fine 200 mesh screen to remove any large clumps of cells or tissue.

Ficoll separation was performed (modification of the method by Davidson and Parish, ref. 93) to remove erythrocytes and dead cells from the spleen cells. The single cell suspension, at r.t., was then layered carefully onto the appropriate volume of Ficoll-metrizoate, maintaining a sharp Ficoll/medium interface. For 3-4 spleens in 15 ml medium, 10.5 ml Ficoll-metrizoate was placed into a 50 ml polypropylene centrifuge tube. For 1-2 spleens in 5 ml medium, 4 ml Ficoll-metrizoate was placed in a 12 ml tube. The cells were centrifuged at 4,200 r.p.m. for 15 min using a swinging bucket HB-4 rotor in a Sorvall RC2-B centrifuge at r.t.

The white cells were removed carefully from the interface and washed in medium at least twice. The first wash at 1,400 r.p.m. (~450g) and the second wash at 1,200 r.p.m. (~350g) were performed in

an International Centrifuge, Model PR-J (~450g) to pellet the cells through any Ficoll inadvertently removed with the cells. For every spleen equivalent, the cells were finally resuspended in 1 ml and counted to determine the concentration and the viability with Trypan Blue exclusion (final 0.1% Trypan Blue).

Preparation of cell-free extracts

1. Sonication

The splenic cells were disrupted by sonication for 2.5 min. For this purpose, the narrow probe of the sonicator was inserted into a polypropylene tube containing the cell suspension, which was kept in an ice bath on a jack platform at 50% maximum energy (50W) of a Biosonik sonicator- Bronwill Scientific, Rochester, NY. The cells were then centrifuged at 10,000 r.p.m. for 10 min to remove the debris. At least 99% of the cells were disrupted by this procedure.

2. Cavitation

Spleen cells were treated once with Tris-NH_4Cl to remove erythrocytes. The cells were then placed in a tightly closed cylinder (Unité d'Immunocytochimie, Institut Pasteur, Paris, France) under 40 atm. pressure for 20 min. The pressure was released suddenly by opening the stopcock underneath the cylinder, at which time the disrupted cells were collected and checked under a microscope for the extent of disruption. The crude extract of cells was obtained after centrifugation at 10,000 r.p.m. for 10 min. Less than 1% of the cells appeared to be intact after this procedure.

3. Freezing and thawing

A cell suspension at 1×10^{8}-cells/ml medium was exposed to 3 cycles of freezing (at -70°C in a dry-ice/acetone bath) and thawing (at 37°C) in order to disrupt the cells. This method consistently resulted in at least 99% disrupted cells. The suspension was centrifuged as above to remove the cell debris.

Immunization

Solution A: 10% $AlK(SO_4)_2$ in ddH_2O w/v

Suspension B: 2 mg/ml of $Al(OH)_3$ for absorption of antigen onto the gel

1. Solution A was diluted 5-fold in PBS, followed by the addition of 1 drop/5 ml of phenol red (0.1% solution) and 1N NaOH until the solution began to turn from yellow to pink. At that point, 0.25N NaOH was carefully added until a stable pink-orange colour developed; it was important that the solution did not turn dark pink, since this would have indicated a conversion of $Al(OH)_3$ to sodium aluminate. The optimal pH was 7.2-7.4.

The 50 ml centrifuge tube was topped with PBS and the precipitate washed 3 times with PBS in order to remove the soluble by-products of the reaction [$2AlK(SO_4)_2 + 6NaOH \longrightarrow 2Al(OH)_3 + 3Na_2SO_4 + K_2SO_4$]

Suspension C: Precipitation of antigen

2. To the appropriate concentration of antigen in a given volume (usually 2 ml) was added an equal volume of Solution A., following the same procedure as above to produce $Al(OH)_3$ while simultaneously trapping antigen in the precipitate. The precipitate was washed at 2000 r.p.m. 3 times with PBS and resuspended in the original volume of the antigen (i.e. 2 ml).

3. Suspension C was diluted with Suspension B to obtain the desired amount of antigen per 0.5 ml for injection into mice.

Passive cutaneous anaphylaxis (PCA)

1. The antiserum to be tested was serially 2-fold diluted in PBS, so that the expected titer was in the middle of a range consisting of 6 dilutions. Each of 6 sites in a line on the back of a male Long-Evans Hooded rat received 50 μl of each of the diluted solutions, injected intradermally (in descending order from the highest dilution at the top to the lowest dilution at the bottom).

2. Each site to be injected had been marked as a dot with a red felt pen on the back of the rat. At least 6 hrs was required for binding of the IgE antibodies to the mast cells in the skin, and for the diffusion of IgG_1 antibodies out of the sensitized sites before challenge. The rat then received i.v. into the penal vein the appropriate antigen (1 ml of a 1:1 solution of 2 mg/ml antigen and 1% Evan's Blue in PBS). The antigen chosen (DNP-NGS, OA, Fl-goat gamma globulin, NP-HGG, NIP-HGG or TM-BSA) was appropriate to the antigen used as immunogen in the mice being tested for IgE antibodies.

3. Twenty minutes after challenge the rat was sacrificed under ether, the back was skinned and the endpoint of titration of each antiserum corresponded to the highest dilution which gave a blue spot of at least 5 mm in diameter. In cases where spot diameters were greater than 5 mm at the endpoint, the diameter was taken as the average of the horizontal and vertical measurements. Furthermore, from duplicate tests of the same antiserum on two different rats, an average value of the spot size at the endpoint was determined.

Instead of reporting 2-fold dilutions as endpoints, this author also took into account the spot size at the endpoint which would further differentiate the titers of the antisera. The dilution 2-fold above a given endpoint was assumed to have a spot of 0 diameter. Then a value was sought for the dilution of this antiserum which would have resulted in a spot of exactly 5 mm. On a graph of spot diameters (y-axis) vs. the $\log_{10}$ of the reciprocal of the dilutions (x-axis), a straight line was drawn to represent the decrease in spot diameter (change in "y") as a function of the increase in "x" between one dilution and the next. Where this line intersected y= 5mm was taken as the actual dilution which would have given a spot of 5 mm diameter (i.e. the endpoint dilution). Only a 4-fold difference in titer between experimental and control groups of mice was considered significant, since the same antiserum tested on two different rats often resulted in a normal variation of 2-fold in the endpoint dilution (data not shown).

Bleeding of mice

Whole mice in a plastic cage were warmed under a heat lamp, and bled by nicking the tail at the most distal point possible without a previous scar proximal to it, so that subsequent bleedings could be performed at successively more proximal points. A shallow nick was made transversely underneath the tail so as to cut an artery and collect at least 12 drops of blood from each mouse. The sera of mice from a given experimental group (3-4 mice) were pooled.

Plaque-forming cell assay

1. Coating SRBC

SRBC in Alsever's solution (10 ml) were washed 5 times in normal saline (0.9%) and after the 5th wash the packed volume was measured. The SRBC were resuspended in 5 times their packed volume of 0.15M saline, 0.14M borate, pH 8.8 (BBS= borate-buffered saline). Ten ml of this 20% solution was reacted with NIP-*O*-succinimide in a 50 ml plastic centrifuge tube. Twenty mg NIP-*O*-succinimide was dissolved in 2 ml of dimethylformamide. Before beginning the reaction, a solution of 1.2 mg/ml glycylgycine in 50 ml of PBS was prepared for stopping the reaction. The NIP-*O*-succinimide was added dropwise with a finely tapered Pasteur pipette (using all 2 ml) while vortexing the SRBC gently. About 5 drops were added per second.

The reactants were allowed to stand for 6 min at r.t., except for one swirl at 3 min. The reaction was stopped by the slow addition of cold glycylglycine in PBS. The cells were washed extensively with PBS until the supernatant was clear of a reddish tinge due to lysed SRBC. Finally, the coupled SRBC were resuspended in 4.3 times their own volume for the PFC assay.

2. Preparation of slides

The PFC assay was a variation of the Cunningham assay (84, 85). The slides were wiped clean with a lint-free gauze soaked in 70% denatured ethanol and were separated from each other with double-sided tape on both ends and in the center so that the assembly of two slides resulted in two microthin chambers for filling with the AFC and target cells.

3. Plaque-forming cell assay

Spleen cells were obtained from experimental groups of mice. The spleens were homogenized in MEM-HEPES, washed once and resuspended in 1 ml Tris-NH_4Cl,pH 7.2, for each spleen equivalent. The spleens were left in this buffer for 5 min to lyse the SRBC, then the tubes were filled with MEM and the cells washed twice before resuspending in MEM containing 5% FCS.

NIP-absorbed Guinea Pig Complement (GPC), i.e. GPC from Cedarlane previously absorbed with NIP-SRBC at 4°C for 20 min, was kept on ice for the assay. For PFC to SRBC, Hemo-Lo GPC (Cedarlane) was used at a ¼ dilution. Three additional 10-fold dilutions of the original spleen cells totaling 2 ml each were prepared for subsequent mixing with other reagents in microtiter wells. The sheep anti-mouse Ig (see pg.29) was added to develop IgG plaques. The spleen cells, NIP-SRBC and complement were mixed as follows:

1.NIP-SRBC control in the absence of C′ and spleen cells

2.NIP-SRBC control with C′ but still without spleen cells

3.

IgM	IgM + IgG
50 μl MEM- 5%FCS	25 μl MEM- 5%FCS
25 μl NIP-SRBC	25 μl anti-MIgG
100 μl spleen cells	25 μl NIP-SRBC
25 μl C′	100 μl spleen cells
	25 μl C′

Duplicate chambers of a Cunningham slide were filled by capillary action with the different mixtures and sealed with a melted mixture of 70% Parrafin wax and 30% vaseline and the slide was then incubated for 1 hr at 37°C. In the meantime a cell count was taken for the

cell suspension of highest concentration from which all other dilutions had been made.

The plaques were enumerated within the lawn of NIP-SRBC, recording the numbers in the duplicate chambers separately. Pseudoplaques, which were caused by aggregates of SRBC which released antibody into the environment, were not counted. A true plaque was a hole in the lawn of target cells, in the center of which was a translucent lymphocyte, larger than the erythrocytes, which was presumably the antibody-forming cell.

The IgM PFC response was calculated from the dilution which gave between 20-200 "direct" plaques in each chamber of the slide. Knowing that each slide received 100 μl of spleen cells at a given dilution, the final calculation was made by multiplying the total number of plaques per slide by the following factors:

1. x10 for number of plaques/ ml of the cell dilution
2. by the ratio of 10^8/cell number in 1 ml of a given cell dilution.

This calculation corresponded to the number of plaque-forming cells (PFC)/ 10^8 spleen cells.

To arrive at the correct number of IgG plaques, the IgM plaques not inhibited by the anti-Ig (shown previously to be 54% of direct plaques) were subtracted from the total plaques (IgM + IgG) obtained on incubation of the AFC with the anti-Ig antibodies.

<u>Nylon wool enrichment of T cells from the spleen</u>

This method was a modification of that developed by Julius *et al.* (96). Nylon wool (obtained in bulk packages from Baxter-Travenol) was

first treated with 0.1N HCl in a large flask at r.t. for 24 hrs. This acid treatment was only required for previously unused nylon wool. It was then boiled in ddH_2O for 3 hrs on 3 consecutive days, changing the ddH_2O between each day for overnight incubation. The clean nylon wool was then dried at 37°C and teased with a carding brush to separate the strands. For each 20 ml syringe to be used for filtering the spleen cells, 2.4 gms recycled nylon wool was gently packed into the column. The column was filled using a syringe and connecting tube from the bottom up with warm MEM-5% FCS, avoiding the formation of air pockets. The column was maintained in a 37°C incubator for 1 hr prior to use.

Ficoll-purified SC were resuspended in MEM-5% FCS at $1x10^8$ viable cells/ml and incubated on the column as follows:

1) 3 ml of the cell suspension was applied to a nylon wool column and allowed to completely enter the column.

2) 2 ml of warm medium was added to the column which was then incubated for 15 min at 37°C.

3) Step 2) was repeated 3 more times.

4) 45 ml of warm medium was passed slowly through the column, eluting any non-adherent cells (referred to hereafter as NW T cells), at a rate of 3 ml/min. The NW T cells contained no more than 2.5% sIg+ by the Protein A-SRBC rosette assay. The recovery of the cells was 10-15% of the original SC applied to the NW column.

Positive selection of spleen T cells from plates coated with anti-MIgG

1. Plastic petri dishes (Allied Fisher Scientific Co., Cat. # 8-757-12, 100x15 mm) were coated with 2.5 ml each of a solution of 50 or 100 μg/ml of affinity purified goat anti-MIgG antibodies or

globulin precipitate obtained at 35% SAS of a sheep anti-NMS; both of these antibody preparations were shown to react strongly on Ouchterlony tests with MIgG. The plates were left overnight at 4°C for at least 12 hrs and washed 3 times with PBS before addition of 5% FCS in PBS to the plates. The plates were allowed to stand at r.t. for 1 hr before use, so as to block any remaining non-specific protein-binding sites.

2. The plates coated with 50 μg/ml of anti-Ig antibodies were rinsed once more with PBS before applying 5 ml of cells at $2x10^7$/ml in MEM-5%FCS. The plates were mixed gently every 15 min for 1 hr and then the non-adherent cells were poured off after gentle swirling followed by 2 additional washes with MEM. The plates were then flooded with 5 ml of 10% NMS-2%FCS in MEM to nudge off the sIg+ (B) cells (adherent fraction), which could be easily removed by gentle pipetting with a Pasteur pipette. The recovery of the sIg+ cells was about 50% of the original SC plated.

The non-adherent cells were centrifuged and resuspended in MEM-5% FCS, so that for every 3 plates during the first incubation 1 plate (coated with 100 μg/ml anti-Ig) was used for another ½ hr incubation, with gentle mixing after 15 minutes. The non-adherent cells displaced by gentle swirling, which were poured off from these plates, were considered sIg-, since by Protein A-SRBC/RaMIg rosetting they contained less than 2% sIg+ cells, compared to 50% sIg+ cells for SC. The recovery of sIg- cells was between 18-25% of the original SC plated.

Protein A-SRBC rosette assay for detecting sIg+ cells

Coupling SRBC to Protein A

Ten ml SRBC in Alsever's Solution were washed 4 times with 0.9% saline. The SRBC were finally resuspended to $1x10^{10}$/ml. A 1/50 dilution of $CrCl_3$ (0.1% in saline)[1] was prepared (0.3 ml $CrCl_3$ + 14.7 ml saline). Ten ml was added dropwise to 1 ml SRBC + 0.5 ml Protein A (1 mg/ml). The reaction was continued for 60 min at 37°C, with mixing every 5 min. The SRBC were washed 5 times with PBS to stop the reaction. The SRBC-Protein A were resuspended in 2 ml MEM ($5x10^9$/ml).

Coating lymphocytes with anti-Ig antibodies

Spleen cells were purified on Ficoll and resuspended at $4x10^7$/ml in MEM. For every 1 ml of cells, 0.1 ml of rabbit anti-mouse Ig antibodies(Protein A-purified;1 mg/ml) was added. The Ab was allowed to bind on ice for 30 min with occasional stirring and the cells were washed once with MEM to remove unbound Ab. The cell concentration at this point was about $4x10^7$/ml.

Combining SRBC-Protein A with spleen cells coated with anti-Ig

A 0.2 ml pellet of SRBC-Protein A and 0.2 ml PBS were mixed and 0.2 ml of this mixture was added to 0.2 ml spleen cells. The mixture was centrifuged at 500 r.p.m. for 4 min in a PRJ centrifuge. The cells were left packed at r.t. for 30 min and resuspended vigorously with a Pasteur pipette to isolate the rosettes. They were then diluted ten times with MEM and finally twice with crystal violet (0.1 ml + 0.1 ml). They were left at r.t. for 5-10 min and the rosettes counted under a microscope (4 SRBC/lymphocyte minimum for a rosette).

Precipitation of suppressive extract at 50% SAS

Five ml of sonicated extract from SC of tolerized mice was placed in a 50 ml centrifuge tube. Five ml of SAS at 4°C was added dropwise with constant stirring. The stirring was continued for 30 min, whereupon the precipitate was centrifuged at 15,000 r.p.m. for 10 min and the supernatant removed. The precipitate was redissolved in 2.0 ml of ddH_2O. Both the precipitate and the supernatant were dialyzed against PBS, pH 7.2, with 3 buffer changes. The supernatant was also concentrated overnight to less than 5 ml. Finally, both fractions were made up to 5 ml with PBS.

Treatment of SC with anti-Thyl.2 antiserum + C′

1. Anti-Thyl.2 was stored as a 1/20 dil. in 50%FCS/MEM, in 150 μl aliquots at -70°C. This had to be diluted with MEM 12.5x for a 1:250 dilution. The diluted anti-Thyl.2 antiserum was added dropwise while stirring in a 1:1 ratio with SC at $2x10^7$ or $1x10^7$/ml. The antibodies were allowed to bind for 45 min at 4°C. The cells were then centrifuged and the supernatant removed. Finally the cells were resuspended in a ten-fold dilution of Lo-Tox Rabbit Complement (Cedarlane) and incubated for a further 45 min at 37°C. Two control groups were included. One received no Ab or C′, the other only C′.

2. The cells were enumerated for dead cells by counting at least 100 cells for each sample in Trypan Blue. The total % of T cells lysed was taken as the % of dead cells(Ab +C′)- 2/3 % of dead cells(C′), since about 2/3 of non-specifically lysed SC could be expected to be non-T cells.

Preparation of B6nIg

A precipitate of B6NMS at 40% of saturation with ammonium sulphate was prepared as follows:

B6NMS was clarified by centrifugation (10,000 r.p.m., 5 min). To the serum (22.3 ml) was added slowly at 4°C a solution of SAS (14.9 ml) with continuous stirring until 40% saturation was reached. The precipitate was allowed to form for some hrs, preferably overnight. Incubation in an ice bucket overnight is essential when precipitating Ig's from dilute solutions (<1mg/ml). The slurry was mixed and centrifuged at 10,000 r.p.m. for 5 min. Finally, the precipitate was dissolved in a minimal amount of ddH_2O (11 ml), and dialyzed twice against 0.15M NaCl at 200 times the volume and once against Tris-HCl, pH 8.0 (0.05M Tris, 0.15M NaCl, adjusted to pH 8.0 with HCl).

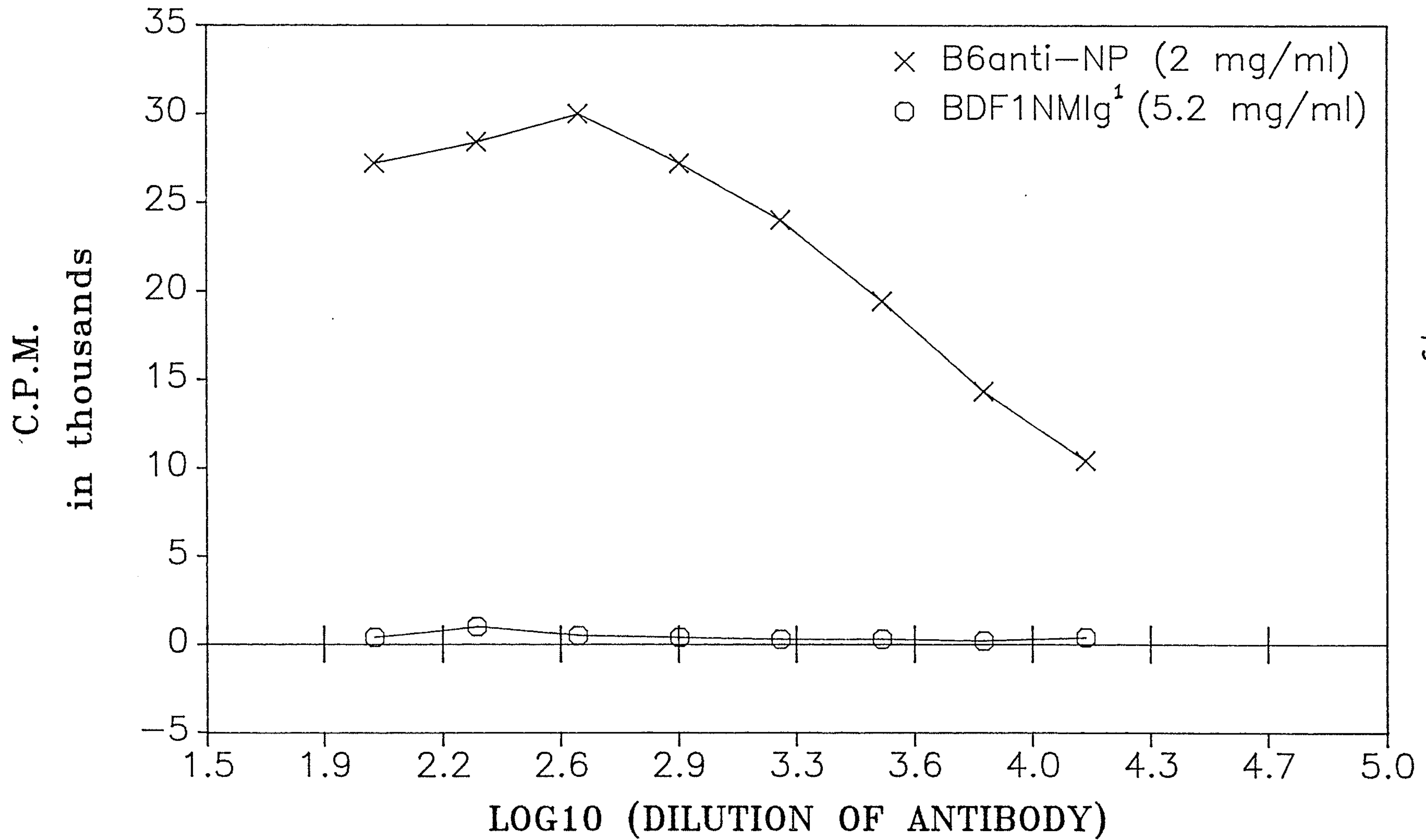
The final sample of B6nIg was at 11.3 mg/ml in 12.5 ml. This sample was applied to ACA-34 (2.6 cm x 90 cm) in a Pharmacia K 26/100 column. The IgM and IgG peaks were pooled, concentrated on an XM-100A membrane and rerun on the same column in order to obtain fractions relatively free of contamination by other serum components. The final proportions of IgM and IgG in the total Ig collected were 31% and 69%, respectively. The same proportions were kept when IgM and IgG (B6nIg) were coupled to Seph 4B.

Preparation of B6anti-NP

One hundred C57BL/6 mice were immunized i.p., each with 100 μg $NIP_{9.5}$-OA + 10^9 B.pertussis + 1.25 mg $Al(OH)_3$. The mice were bled on d14, 17 and 20, and the bleedings pooled. The pooled antiserum was applied to a column of NP_{14}-BSA-Seph 4B in stages according to the capacity of the column (20 ml serum at a time onto a 25 ml column); 0.1M EDTA, pH 8.0 was included to prevent non-specific binding to the column, with a 1:9 ratio of EDTA:antiserum (31). The buffer for running the sample was PBS in the presence of 0.5% Tween-20. The sample was applied to the column and after 1 hr at r.t., elution was begun. The unbound material was passed through the column and elution was continued until the O.D. reading returned to background. The bound NP-specific antibody was then eluted with 0.005M NP-ϵ-amino-caproic acid conjugate (Biosearch) in running buffer. The eluted peak fractions representing affinity-purified B6anti-NP antibody from 5 separate column runs were pooled, dialyzed against PBS to remove free NP-ϵ-amino-caproic acid and concentrated to 2 mg/ml.

Solid phase radioimmunoassay

The specificity of the B6anti-NP antibody was confirmed by a solid-phase RIA, using as second antibody an immunologically purified ^{125}I-labeled sheep anti-mouse Fab on NP-BSA coated plates (see Fig. 3). The wells of a 96-well U-bottom plate were coated overnight at r.t. with NP-BSA (50 μg/ml in 0.05M $NaHCO_3$-Na_2CO_3, pH 9.0) and washed 3 times with PBS. A 10% solution of BSA in the $NaHCO_3$-Na_2CO_3 buffer was used to block the remaining sites, for 3 hrs at r.t. (200 μl/well). The wells were washed 3 times with PBS. Dilutions of



[1] Immune-purified on GantiMIgG-4B

the antibody were added to the wells (200 μl/well) and incubated for 2 hrs at r.t., followed by a wash. Finally, the second antibody (radiolabeled sheep anti-mouse Fab antibodies) was added in assay buffer ($1x10^6$ c.p.m. in 150 μl) and incubated for 2 hrs at r.t. The plates were washed and dried before counting the wells in a Beckman gamma counter.

Use of protein-Seph 4B as an immunosorbent

Coupling of protein to Sepharose 4B

Packed Sepharose 4B gel (100 ml) was centrifuged and defined at 400 r.p.m. for 10 min using ddH_2O to define 3 times. An equal amount of ddH_2O was added, the slurry was transferred to the fume hood and stirring was initiated. For each ml of Seph 4B, 30 mg CNBr dissolved in acetonitrile was added with vigorous stirring (2 gm CNBr/3 ml acetonitrile). The pH was immediately adjusted and maintained at 10-11 with 4N NaOH and the temperature maintained at 20°C (by the addition of crushed ice when required) for 10-15 min to allow the reaction to go to completion.

The gel was poured onto a Buchner funnel and washed with a large volume of cold $NaHCO_3$-Na_2CO_3 buffer (0.05M; pH 10.0). It was then transferred with a minimum volume of buffer (½ volume of gel) to an Erlenmeyer flask in an ice bath. With stirring, protein (NIP-BSA, NP-BSA, B6anti-NP or B6nIg) was slowly added at 100 mg protein/100 ml packed gel (protein was dissolved in pH 10 buffer at ½ the gel volume). The slurry was stirred in the cold overnight.

The gel was filtered and the filtrate collected. The gel was

washed with a small volume of cold buffer and the wash collected The wash was added to the filtrate, and the amount of protein in the combined volume was determined spectrophotometrically at 280 nm for the amount coupled[1] (if the filtrate was turbid, it was first centrifuged at 2000 r.p.m. for 15 min). The gel was sucked dry, washed twice with buffer, and resuspended in gelatin (4 mg/ml, pH 10) for 3 hrs at 4°C with gentle stirring to block excess reactive sites. The gel was then filtered and washed with ddH_2O.

The coupled gel was resuspended in 4M guanidine-HCl-0.25M sodium acetate buffer, pH 4.0 for 10 min at r.t. to strip it free of any adsorbed protein for future adsorptions. It was washed in ddH_2O, then PBS, and finally equilibrated in an equal volume of PBS, pH 7.4 + 0.1% NaN_3.

Binding of N^{125}IP-PVA to B6anti-NP-Seph 4B

When N^{125}IP-PVA was incubated batchwise with B6anti-NP or B6nIg coupled to Seph 4B, more than 96% of the conjugate was bound. 0.5 ml gel was mixed with 0.5 ml N^{125}IP-PVA, rotated for 1 hr at 4°C, centrifuged and the supernatant separated from the gel. The estimated capacity of the B6anti-NP column for unlabeled NIP-PVA was 30 μg/ 0.5 ml gel. Therefore, a slight excess (40 μg) of unlabeled NIP-PVA was added with 2,000 c.p.m. of N^{125}IP-PVA to each fresh gel (Expts. 1 and 2). In Expt. 3, gels were pre-incubated with 500 μg $PVA\text{-}NH_2$ in 1 ml

[1] B6anti-NP: 0.71 mg/ml
B6nIg: 0.95 mg/ml
NIP-BSA: 0.75 mg/ml

before adding 0.5 μg of $N^{125}IP$-PVA, to test for the effect of unlabeled PVA on the non-specific binding of NIP-PVA.

Expt.	% Bound		
	B6anti-NP	B6nIg	Specific for B6anti-NP
1.	64	26	38
2.	60	32	28
3.	92	62	30

Ultrafiltration

Amicon filter units (10 ml capacity) were used for fractionation of samples on Amicon ultrafiltration membranes (with a diameter of 25 mm) and various pore sizes: PM-10 (10,000 M.W. cut-off), XM-100A (100,000 M.W. cut-off) and XM-300 (300,000 M.W. cut-off). The filtrate included one wash of the membrane and the retentate contained the material which did not pass through the filter. During filtration the membrane was never allowed to go to dryness. For the purpose of sample concentration, only the retentate was collected.

Pulsing SAC with F/T_E from spleen T cells of normal or tolerized mice.

This procedure was a modification of the method by Cowing *et al.* (115). Spleen cells from 14 normal C57BL/6 mice were purified on Ficoll and resuspended in RPMI containing 20 mM HEPES, 10%FCS, 2mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (complete medium). A volume of 5 ml containing $5x10^7$ SC was applied to

each glass Petri plate and incubated for 2 hr in a 37°C incubator with 5% CO_2. The non-adherent cells were removed from the plate by gentle swirling and pouring. A volume of 10 ml fresh warm medium was added to the plate, which was incubated further at 37°C for 2 hr. The nonadherent cells were again removed from the plate with 3 washes of warm medium, with the first wash requiring gentle pipetting with a 5" Pasteur pipette to remove loosely adherent cells as well. The plate was then flooded with 5 ml of Versene at 1:5,000 (0.6 mM EDTA solution provided by Gibco Labs.) and incubated at 37°C for 15 min to dislodge the adherent cells. Adherent cells were then washed off with vigorous pipetting, centrifuged and resuspended in complete medium. The recovery of original SC in the form of non-adherent cells was 75% and of adherent cells was 11.8%.

The adherent cells (at 3.3×10^7/ml in 5 ml) were then incubated for 6 hrs at 37°C in 5% CO_2 on a Teflon insert of a glass Petri plate. This step was included to allow the cells to recover from the EDTA treatment. About 67% of cells were recovered from the Teflon (1.1×10^8 in 1 ml) and resuspended in RPMI containing 0.1 mM HEPES, 2mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Simultaneously, F/T_E of spleen T cells from both normal and tolerized mice were obtained in the same medium.

The adherent cells were divided into two separate groups such that 1×10^7 adherent cells would be available for pulsing with the F/T_E from 1×10^8 cells (from either the tolerized or control group of donor mice). The cell pellets were resuspended in F/T_E and pulsed for 2 hrs, with gentle rocking in a 37°C incubator without added CO_2. After centrifugation, the supernatant was removed carefully from the

cells, which were washed once with the RPMI + 20 mM HEPES before resuspension in the same medium at a density of $7x10^6$ cells/ml. Each recipient mouse was injected i.v. with $2x10^6$ cells in a volume of 0.3 ml.

The enrichment for Mϕ in the adherent population was tested by the non-specific esterase staining method for macrophages (measuring α-naphthyl acetate esterase activity) in a modification of the procedure by Koski *et al.* (116). The SAC's after pulsing with F/T_E from tolerized mice) were 25% positive for esterase, comparing favorably with a previous experiment for which 30% of SAC's just before pulsing were esterase-positive. The latter experiment also demonstrated only 1.4% esterase + cells among the non-adherent spleen cells obtained after the first 2 hr incubation on glass, which implied that most adherent cells were removed by this step. By the same staining method, SC of BDF_1 mice were shown to be 10% esterase-positive (macrophages), which concurs with the report by Springer *et al.* (94) that 8% of spleen cells, most likely macrophages expressed Mac-1, a cell-surface antigen found on mononuclear phagocytes, granulocytes and NK cells.

Non-specific esterase staining.

The following solutions were prepared ahead of time:

Pararosaniline-HCl (4%)- 4 gm of pararosaniline HCl, 20 ml concentrated HCl and 80 ml ddH_2O, mixed and filtered with a Whatman type 541, 9.0 cm diameter.

Methyl green (counterstain)- a 1% solution in ddH_2O, also filtered.

On the same day:

a) 1.5 ml 4% Pararosaniline-HCl was added to 1.5 ml 4% $NaNO_2$ (fresh weekly).

b) 2.5 ml of ethylene glycol monomethyl ether was added to 50 mg α-naphthyl acetate and mixed in a capped tube by inversion.

Each of a) and b) were added to 44.5 ml PBS, which was mixed after each addition with a magnetic stirrer. The pH was adjusted to 6.1 with 1N NaOH and the stain filtered.

The cell smears (cells at $3\text{-}5 \times 10^6$/ml) on slides fixed for 30 sec with 2% glutaraldehyde in PBS were incubated in the above substrate for 50 min at r.t., rinsed in ddH_2O and incubated in methyl green counterstain for 15 sec. The cells stained with reddish-brown granules were enumerated as esterase +.

Irradiation of mice or cells

A ^{60}Cobalt source was used for irradiation of mice or cells. Briefly, a Theratron F Cobalt 60 Unit, calibrated by the Physics Department of the Manitoba Cancer Treatment and Research Foundation, provided a measurable dose of irradiation, dependent on the distance between a plexiglass cage containing the mice and the ^{60}Co source.

RESULTS

I. Tolerance induction by hapten-PVA treatment and transfer of suppression by splenic cells of tolerized mice or by extracts of these cells

Earlier work from this laboratory (60) indicated that i.p. injection of 1 mg of DNP-PVA into BDF_1 mice resulted in suppression of the DNP-specific IgE primary and secondary responses in these mice. In two experiments in the present study mice treated with DNP-PVA three to four weeks after priming with an immunogenic dose of the DNP-OA in $Al(OH)_3$ also exhibited hapten-specific suppression of the secondary response to DNP-OA. Thus DNP-PVA suppressed an ongoing response to DNP but not to the carrier protein OA [Tables 1.a),b)].

Flu-PVA also suppressed a secondary anti-Flu IgE response, whether the Flu-PVA was administered 1 day before or 1 day after the secondary immunization (Table 2). The Flu-PVA most likely acted directly on the Flu-specific B cells, since it would be difficult to envisage that sufficient suppressor T cells could be generated by Flu-PVA given within 1 day of immunization to compete with the anamnestic response of Flu-specific B cells. As reported by Lee and Sehon (48) treatment of BDF_1 mice with $DNP_{1.4}$-PVA induced a form of suppression that could be transferred to syngeneic recipients through the spleen T cells; furthermore, these suppressor T cells were Cy sensitive. Similarly, mice were treated by this author with Flu-PVA alone 7 days prior to sacrifice (Table 3), and the transfer of their whole spleen cells into syngeneic recipients suppressed the primary anti-Flu response in the recipients (Groups 1 and 2), without suppressing the carrier-specific portion of the response. Similarly,

Table 1.a) Suppression of an ongoing response with $DNP_{1.4}$-PVA in BDF_1 mice.

TREATMENT[1]	PCA TITERS					
	anti-DNP			anti-OA		
	d7	d14	d29	d7	d14	d29
1. DNP-PVA on d20	25	1580	235	10	1580	3720
2. ddH_2O on d20	25	1580	2820	10	1580	1620

[1]All mice were primed with 1 µg DNP-OA+$Al(OH)_3$ on d0 and challenged with 1 µg DNP-OA+$Al(OH)_3$ on d21. Before d20, groups 1 and 2 were in the same group.

Table 1.b) Suppression of an ongoing response in BDF_1 mice with $DNP_{1.4}$-PVA.

TREATMENT[1]	PCA TITERS				
	anti-DNP				
	d7	d15	d21	d35	d42
1. DNP-PVA on d27	1412	2820	708	290	320
2. PBS on d27	2560	2820	891	2950	2690
	anti-OA				
	d7	d15	d21	d35	d42
1. DNP-PVA on d27	63	2820	1412	3470	3315
2. PBS on d27	91	1660	1350	3020	3020

[1]All mice were primed with 1 µg DNP-OA+$Al(OH)_3$ on d0 and challenged with 1 µg DNP-OA+$Al(OH)_3$ on d28.

Table 2. Suppression of an ongoing response with $Flu_{2.0}$-PVA in BDF_1 mice.

TREATMENT[1]	PCA TITERS			
	anti-Flu		anti-OA	
	d35	d42	d35	d42
1. Flu-PVA on d27	100	20	3510	3160
2. ddH_2O on d27	810	210	1490	760
3. Flu-PVA on d29	80	25	3320	3160
4. ddH_2O on d29	850	310	850	710

[1]All mice were primed with 1 µg Flu-OA+$Al(OH)_3$ on d0 and boosted with 1 µg Flu-OA+$Al(OH)_3$ on d28.

Table 3. Suppression induced in recipients of spleen cells or splenic extracts of donors treated with $Flu_{2.0}$-PVA 7 days prior to sacrifice.

BDF_1 ♂♀ ⟶ 7 DAYS ⟶ spleen cells (SC) / cell extracts (SC_E)[1]

$Flu_{2.0}$-PVA ↑

TREATMENT OF RECIPIENTS[2] d0	PCA TITERS			
	anti-Flu		anti-OA	
	d15	d22	d15	d22
1. SC(tol)-$6x10^7$	25	20	235	270
2. SC(nl)-$6x10^7$	340	290	90	310
3. SC_E(tol)-$1x10^8$	95	60	270	290
4. SC_E(nl)-$1x10^8$	270	235	80	255

[1]Cells were sonicated (see MATERIALS AND METHODS)
[2]All mice received 1 µg Flu-OA+$Al(OH)_3$ on d1

5 days after treatment of naive mice with $DNP_{1.4}$-PVA their spleen cells could transfer to syngeneic mice a state of nonresponsiveness to DNP (Table 4, Groups 2 and 3). Cells that transferred suppression could also be detected 7 to 14 days or even 22 days after DNP-PVA treatment (Tables 5 and 6, respectively). Furthermore, spleen cells enriched for T cells (i.e., sIg- cells) on goat anti-mouse Ig-coated plates could also transfer the suppression (Table 6). Based on these encouraging results, this project then focussed on the indirect suppressive effects of hapten-PVA conjugates, ensuing from transfer of spleen T cells of donor mice treated with hapten-PVA conjugates. Both IgE and IgG primary hapten-specific responses were examined as putative target responses of this suppression.

To investigate the possibility that suppressor factor(s) were involved in the transfer of non-responsiveness by spleen cells of DNP-PVA or Flu-PVA treated mice, these cells were sonicated to produce extracts. These extracts indeed contained suppressive activity (as shown in Tables 4 and 5 for DNP-PVA and in Table 3 for Flu-PVA). The suppressive factor(s) in these extracts (S.F.) were then subjected to treatments that specifically affected proteins. The S.F. was precipitable with 50% SAS (Table 7), indicating a similarity with some proteins. In the case of spleen T cell extracts containing S.F., treatment at 56°C for 90 min (Table 8) resulted in loss of suppressive activity compared to the MEM control (suppression only 2-fold, compared to the same extracts incubated at 4°C, which gave 4-fold suppression), indicating that it was heat-labile. Fractionation of SC extracts on a calibrated Sephadex G-75 column (Fig. 4)

Table 4. Suppression induced in recipients of spleen cells or splenic extracts of donors treated with $DNP_{1.4}$-PVA 7 days prior to sacrifice.

	TREATMENT OF RECIPIENTS		PCA TITERS (d15)	
	d0	d1	anti-DNP	anti-OA
1.	$2x10^7$ SC		290	1280
2.	$6x10^7$ SC	DNP_3-OA	90	710
3.	$2x10^7$ SC_E	1 μg	235	760
4.	$1x10^8$ SC_E	+$Al(OH)_3$	80	660
5.	no treatment		2950	1580

[1]cells were sonicated (see MATERIALS AND METHODS)

Table 5. Transfer of suppression with whole spleen cells or their soluble extracts at different times after $DNP_{1.4}$-PVA treatment.

	TREATMENT OF DONORS	TREATMENT OF RECIPIENTS[1]	PCA TITERS (d22)	
		d0	anti-DNP	anti-OA
A.	DNP-PVA d-14	$6X10^7$ SC	25	320
	" d-10	"	20	320
	" d -7	"	20	340
	no treatment	"	320	330
	DNP-PVA d-14	$1x10^8$ SC_E	80	340
	" d-10	"	80	365
	" d -7	"	65	340
	no treatment	"	280	320

			PCA TITERS (d14)	
			anti-DNP	anti-OA
B.	DNP-PVA d-10	$2.4x10^7$ SC[2]	30	235
	no treatment	"	330	200
	DNP-PVA d-10	$4x10^7$ SC_E[3]	60	235
	no treatment	"	270	220

[1]All mice received 1 μg DNP-OA+$Al(OH)_3$
[2]Pretreated with 0.02M Tris, 0.75% NH_4Cl, pH 7.2 to lyse red cells
[3]Obtained by cavitation (see MATERIALS AND METHODS)

Table 6. Transfer[1] of suppression by spleen T cells fractionated on Goat antiMIg-coated plates, in BDF_1 mice.

	TREATMENT OF RECIPIENTS[2] d0	PCA TITERS (d15) anti-DNP	PCA TITERS (d15) anti-OA
1.	SC(tol)-6x10^7	220	1410
2.	SC(nl)-6x10^7	3020	1480
3	T(tol)-6x10^7	270	1620
4.	T(nl)-6x10^7	1580	1410

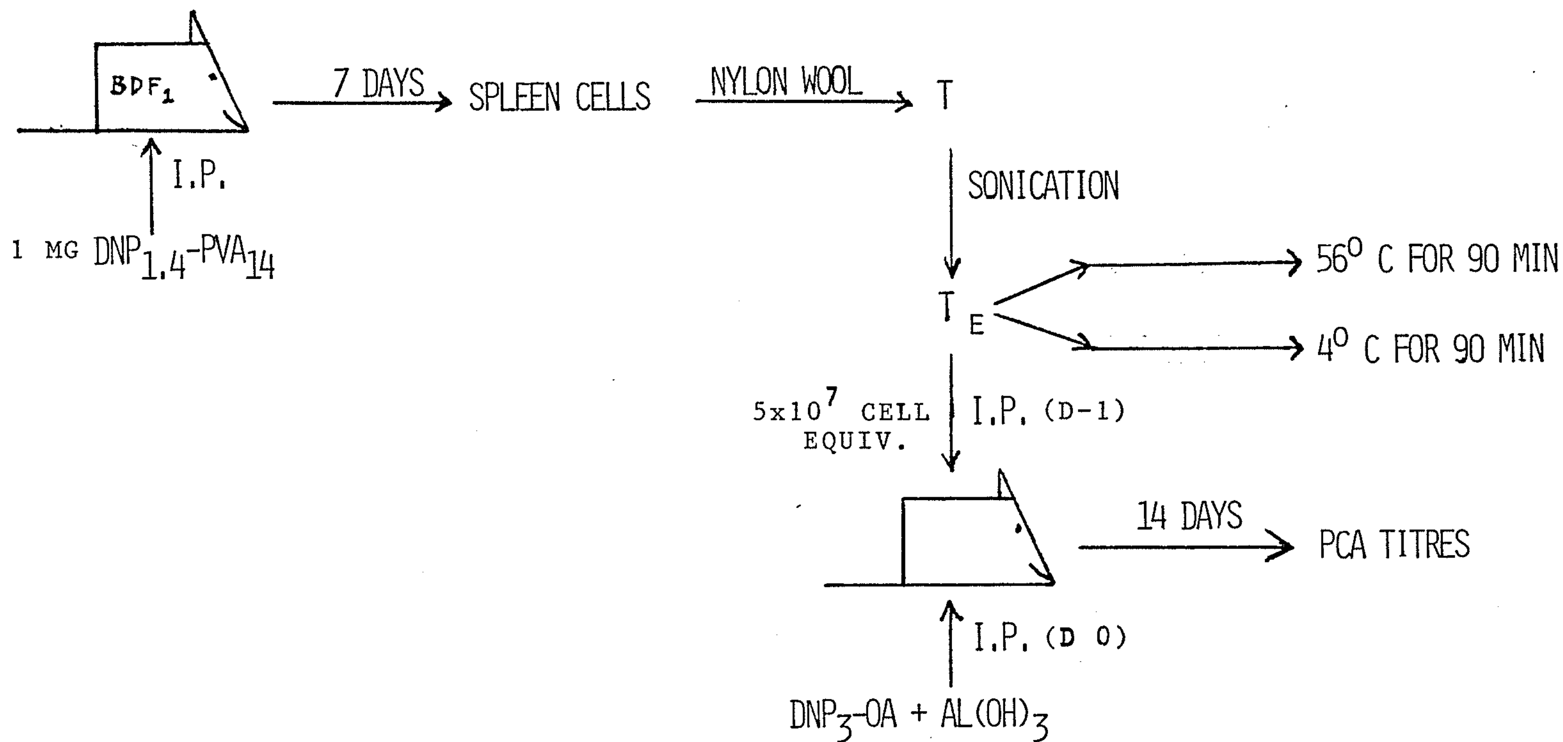
[1] 22d after $DNP_{1.4}$-PVA treatment
[2] All mice received 1 µg DNP-OA+$Al(OH)_3$

Table 7. Treatment of suppressive extracts from BDF_1 mice with 50% SAS.

	TREATMENT OF RECIPIENTS[1] d0	FRACTION AFTER 50% SAS	PCA TITERS (d22) anti-DNP	PCA TITERS (d22) anti-OA
1.	SC_E[2]	precipitate	200	1490
2.	SC_E	supernatant	760	790
3.	No treatment	--	1410	1350

[1] All mice received 1 µg DNP-OA+$Al(OH)_3$
[2] Spleen cells obtained 14 days after treatment of BDF_1 mice with $DNP_{1.4}$-PVA

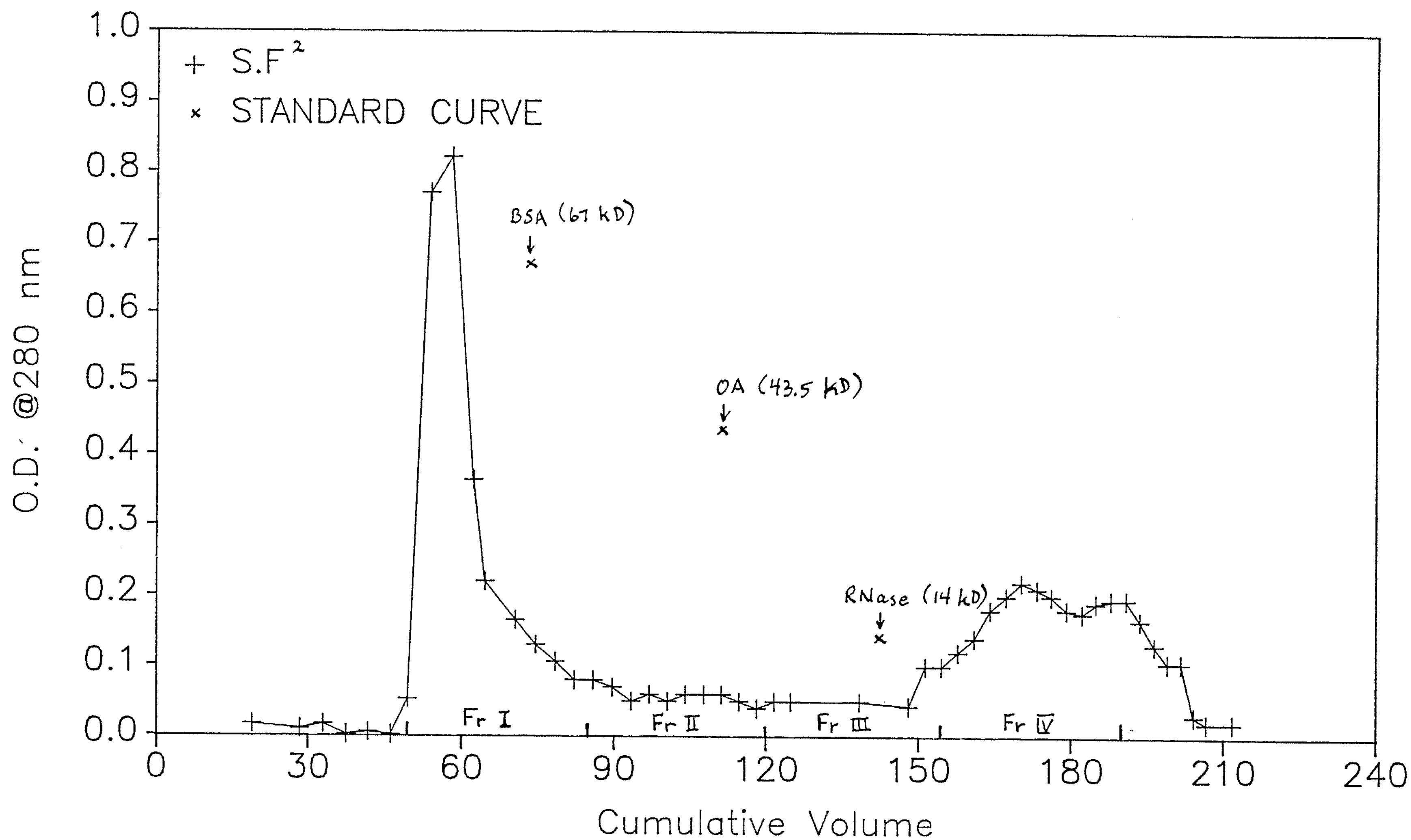
TABLE 8. PARTIAL INACTIVATION OF T_E AT 56^{0} C FOR 90 MIN



TREATMENT OF NORMAL RECIPIENTS	PCA TITRES ANTI-DNP	% SUPPRESSION
CONTROL- MEM	3160	NIL
N_E (4^{0} C)	2820	11
N_E (56^{0} C)	2820	11
T_E (4^{0} C)	794	75
T_E (56^{0} C)	1493	53

N_E= EXTRACT OF NYLON WOOL NON-ADHERENT CELLS FROM UNTREATED DONORS

FIGURE 4
FRACTIONATION OF SUPPRESSIVE EXTRACT ON G-75 COLUMN[1]



1 void volume= 58 ml
2 5x10 cell equivalents in 5 ml

suggested that the molecular size of the S.F. was greater than 60 kD, since the suppressive activity was contained in the void volume (Table 9).

While the above studies were in progress, Mr. John Jeffrey, working with Dr. R.J. Schwenk, was employing NIP-PVA to study the role of the idiotype network in the generation of suppressor cells, because of the dominant NP^b id in the anti-NP response. It was demonstrated that NP- or NIP-PVA conjugates could suppress the IgE responses of BDF_1 mice to NP and NIP (data not shown). As defined in the LITERATURE REVIEW, NP-specific antibodies of C57BL/6 mice exhibit heteroclicity. Therefore, a hybrid strain like BDF_1 (C57BL/6 X DBA/2) would be expected to express this cross-reactivity, since the gene for the NP^b id was shown to be dominant, i.e., Imanishi and Makela (73) reported that the C57 trait was dominant in the F_1 generation of CBA X C57BL/6 mice, since 80% of the F_1 mice exhibited heteroclicity.

It was further shown by Mr. John Jeffrey that transfer of NW T cells from NIP-PVA treated mice could suppress the IgE response. A few preliminary experiments indicated that in order to obtain transferable suppression, NP-PVA had to be followed by NP-OA, the latter presumably expanding potential T_s clones. With such treatment, non-responsiveness could be transferred that affected both NP- and NIP-specific IgE responses to NP-OA (data not shown).

New NP- and NIP-PVA conjugates with a substitution number of 4 haptenic groups/ PVA molecule were prepared by this author to extend these studies and to characterize NP-specific suppressor T cells that might be induced. The IgE responses of BDF_1 mice immunized with

Table 9. Fractionation of SC_E[1] on a G-75 column.

	TREATMENT OF RECIPIENTS[2]	PCA TITERS (d19)	
		anti-DNP	anti-OA
1.	SC_E(tol)	<80	380
2.	SC_E(nl)	380	810
3.	Fr I (G-75)[3]	<80	710
4.	Fr II (G-75)	440	420
5.	Fr III (G-75)	360	270
6.	Fr IV (G-75)	420	380
7.	MEM-HEPES	1490	1580

[1]Spleen cells for the extracts were obtained from mice treated 16d previously with $DNP_{1.4}$-PVA.
[2]$1x10^8$ cell equivalents/ recipient for Gps. 1-6 on d0, followed by 1 µg DNP-OA+$Al(OH)_3$ for Gps. 1-7.
[3]The molecular weight represented by each fraction of the G-75 column was as follows: Fr I, >60 kD; Fr II, 32-60 kD; Fr III, 5-32 kD; Fr IV, <5 kD. The exclusion limit of a G-75 column is 80 kD.

NP-OA were suppressed directly by both NP-PVA and NIP-PVA, as described in Table 10. The cross-reactivity of the target B cells interacting with either NP or NIP on the PVA agrees with recent evidence by Riley and Klinman (65) that the same population of splenic λ-bearing B cells in C57BL/6 mice responds to both NP and NIP. NIP-PVA treatment also led to NIP-specific suppression in two other strains, C57BL/6 and BALB/c (Table 11).

Since previously, spleen cells from mice treated with a combination of NP-PVA and NP-OA could transfer non-responsiveness, one group of BDF_1 mice was treated i.p. with 1 mg of NIP-PVA 3 days and 1 day before NIP-OA in $Al(OH)_3$ i.p. and control mice received i.p. NIP-OA in $Al(OH)_3$. Twenty-one days later these mice were donors for NW T cells injected into recipients immunized 1d later with either NP-OA or NIP-OA in $Al(OH)_3$. Both anti-NP and anti-NIP IgE titers were suppressed in the recipients of spleen T cells from donors treated with NIP-PVA + NIP-OA, compared to the control recipients of spleen T cells from either NIP-OA treated or naive donors (Fig. 5). These results indicated that the suppressor cells were definitely cross-reactive in their effect, since the target B cells specific for both NP and NIP were suppressed, as detected by their IgE responses.

If suppressor cells with idiotypic properties were to be found in the NIP-PVA system, C57BL/6 mice had to be used, for the reason that they exhibited a predominant id in their antibody response to NP. The literature at this time already had references to idiotype-positive suppressor T cells induced by NP-coupled syngeneic spleen cells (113).

Table 10. Direct tolerance of the hapten-specific IgE response by prior treatment with NIP-PVA or NP-PVA in BDF_1 mice.

EXPT.	TREATMENT on d-3,-1	TREATMENT d0	PCA TITERS anti-NP d14	anti-NP d18	anti-NP d23	anti-NP d27	anti-NIP d14	anti-NIP d18	anti-OA d23	anti-OA d27
A.	NP_4-PVA	NP_4-OA[1]	110	280			100	110		
	NIP_4-PVA	NP_4-OA	<40	25			<40	30		
	PBS	NP_4-OA	2950	1860			3160	2820		
B.	NP_{15}-PVA	NP_4-OA[1]			<40	15			310	1350
	ddH_2O	NP_4-OA			270	330			270	733

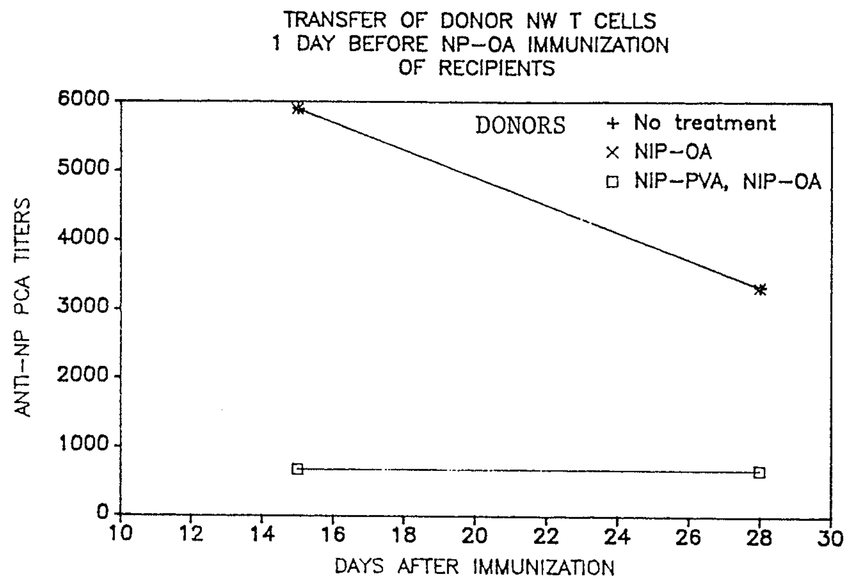
[1] 1 μg in $Al(OH)_3$

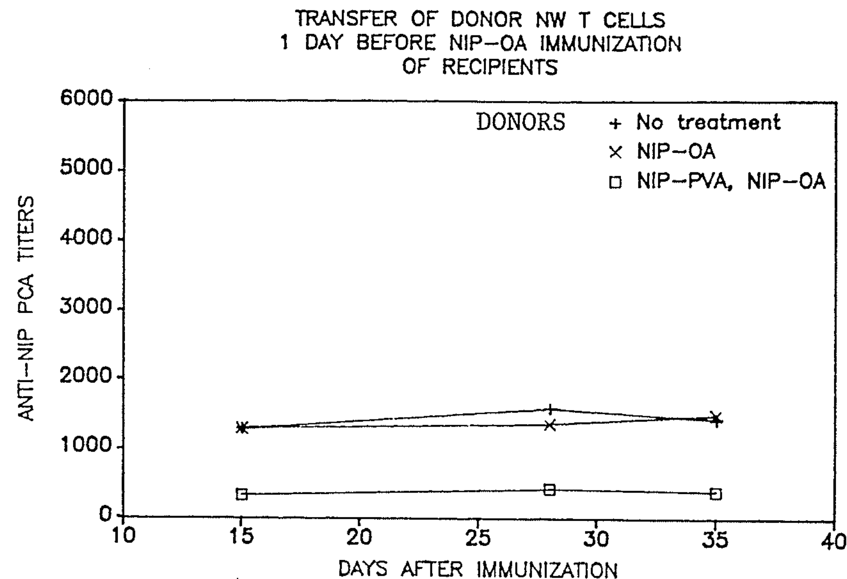
Table 11. Effect of NIP_4-PVA on a primary IgE response to NIP_4-OA.

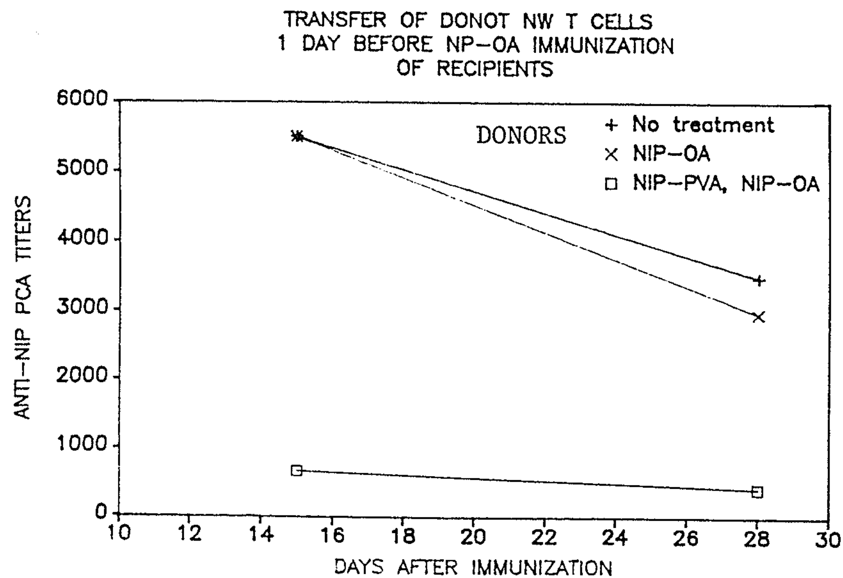
EXPT.	TREATMENT ON d-3,-1	d0[1]	PCA TITERS anti-NIP d11	anti-NIP d14	anti-NIP d15	anti-NIP d22	anti-OA d11	anti-OA d14	anti-OA d15	anti-OA d22
	C57BL/6									
A.	NIP-PVA		<20	<20			55	230		
	PBS		1440	840			120	230		
	BALB/c									
B.	NIP-PVA				120	210			460	380
	PBS				1620	1550			410	360

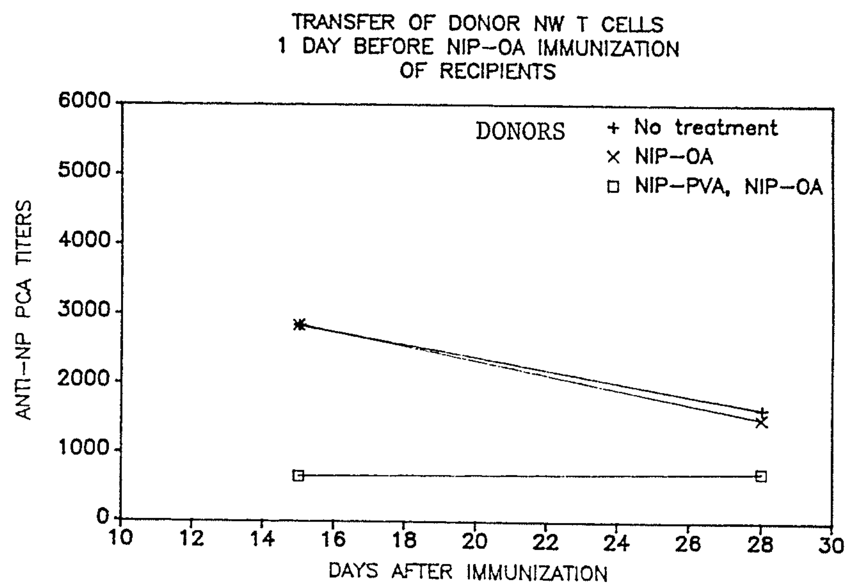
[1] C57BL/6 with 2 μg NIP_4-OA+$Al(OH)_3$ and BALB/c with 10 μg NIP_4-OA+$Al(OH)_3$

Figure 5. Effect of donor NW T cells on anti-NP and anti-NIP IgE Ab's of recipients.









II. Attempts to isolate hapten-specific suppressor T cells and factor(s)

1. Work by Weinberger *et al.* (62) showed that hapten-specific T_s cells could be isolated on dishes coated with hapten-protein conjugates. To obtain putative suppressor cells for isolating on such dishes, donor C57BL/6 mice were treated with hapten-PVA + hapten-OA (NP for 1 expt., NIP for 2 expts.) and 20 days later their spleen T cells were enriched by panning on sheep anti-mouse Ig (ShAMIg)-coated plates, a procedure which leads to removal of sIg+ cells and accessory cells. The T cells of these donors were then subjected to panning on hapten-BSA-coated dishes, but the hapten-binding cells were not suppressive (data not shown). This result could be explained as follows: 1) the suppressor cells transferred were idiotype-specific rather than hapten-specific (from a transducer rather than an inducer subset of suppressor cells); 2) hapten-specific helper cells induced by hapten-OA were isolated along with hapten-specific suppressor cells or 3) there were no T_s cells.

To determine whether suppression could be transferred from the donors before panning, C57BL/6 mice were treated with NIP-PVA and NIP-OA, and their spleen cells were harvested 19 days later. The NW T fraction of these cells did not suppress the IgE response of recipients compared to recipients of the control spleen T cells (Table 12; Group 1 v. 2). In the attempts to isolate hapten-binding cells, there were no suppressor cells present to isolate, because the treatment with immunogen had blocked the transfer of suppression.

To determine whether or not soluble suppressor factors could be extracted from the T cells of NIP-PVA treated donor mice, freeze/thaw

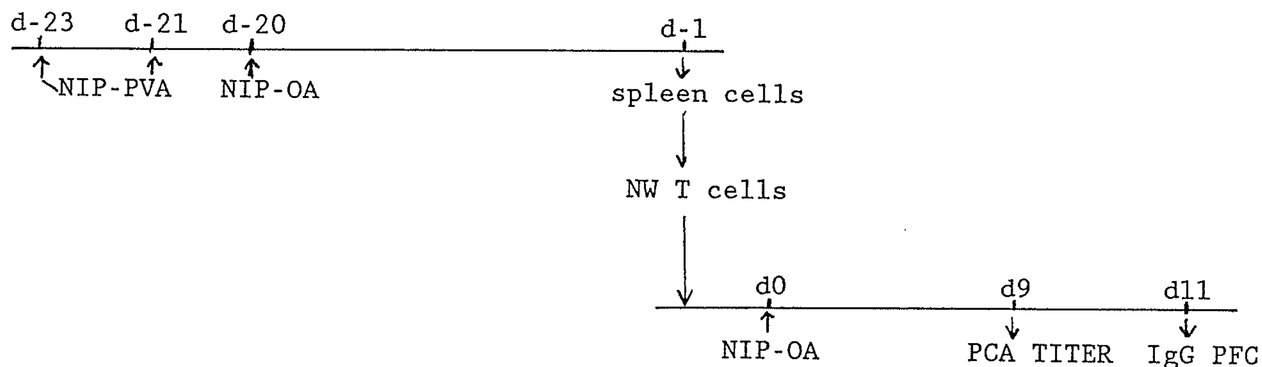


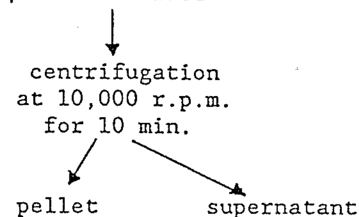
Table 12. Transfer of suppression by NW T cells of NIP_4-PVA treated C57BL/6 donors or by the F/T extracts of these cells.

	TREATMENT OF DONOR	TREATMENT OF RECIPIENT d-1; d0	IgG PFC per 10^8 SC (d11) anti-NP	IgG PFC per 10^8 SC (d11) anti-NIP	PCA TITERS (d9) anti-NP	PCA TITERS (d9) anti-NIP
1.	NIP-PVA+NIP-OA[1]	T; NIP-OA[2]	-	6,500	760	800
2.	NIP-OA	" "	-	28,500	1620	1740
3.	NIP-PVA+NIP-OA	F/T[3]; NIP-OA[2]	-	1,250	<80	75
4.	NIP-OA	" "	-	39,000	460	460

[1] 1 mg NIP-PVA on d-23 and -21 and 1 µg NIP-OA+$Al(OH)_3$ on d -20

[2] 2 µg NIP-OA in $Al(OH)_3$

[3] F/T Extract from T Cells: T cells → freeze and thaw → Crude Extract



This fraction is the non-sedimented component of the F/T extract (F/T_E)

extracts (F/T_E) of the cells were prepared. The F/T_E suppressed both IgE and IgG responses (Group 3 v. 4).

Another experiment was performed to assess the effect of the immunogen on the donor mice treated with NIP-PVA, by dividing them into two groups (Table 13): one receiving NIP-OA (Group 1) and the other no further treatment (Group 3). Each of these donors had appropriate controls without NIP-PVA treatment. In actual fact, NIP-OA treatment prevented the transfer of suppression for the IgE and IgG responses (Table 13, Group 1 v. 2), while NIP-PVA treatment alone resulted in transferable suppression (Table 13, Group 3 v. 4). NW T cells and their F/T extracts had similar effects on the recipients (Groups 1-4), except that in the case of Group 4, the cells suppressed the responses non-specifically, while the extracts did not.

2. Since NIP-OA treatment prevented the transfer of suppression by NIP-PVA treated donor mice, donors were subsequently treated with NIP-PVA, but without NIP-OA. As illustrated by the results in Table 14, their spleen T cells (isolated on ShAMIg-coated plates) as well as the F/T_E of these cells induced NIP-specific suppression without affecting the anti-carrier response [Table 15.a)]. The lack of suppression for the IgM response in Table 14 may have been due to a lower affinity of this response, and hence lower susceptibility to suppression by hapten-PVA conjugates. Nevertheless, from these results, it was inferred that the F/T_E contained a hapten-specific T_sF. The IgG response to NIP was also suppressed by the transferred spleen T cells (Table 14), implying that the suppression was not class-specific.

Hapten-specificity of the F/T_E was further demonstrated by im-

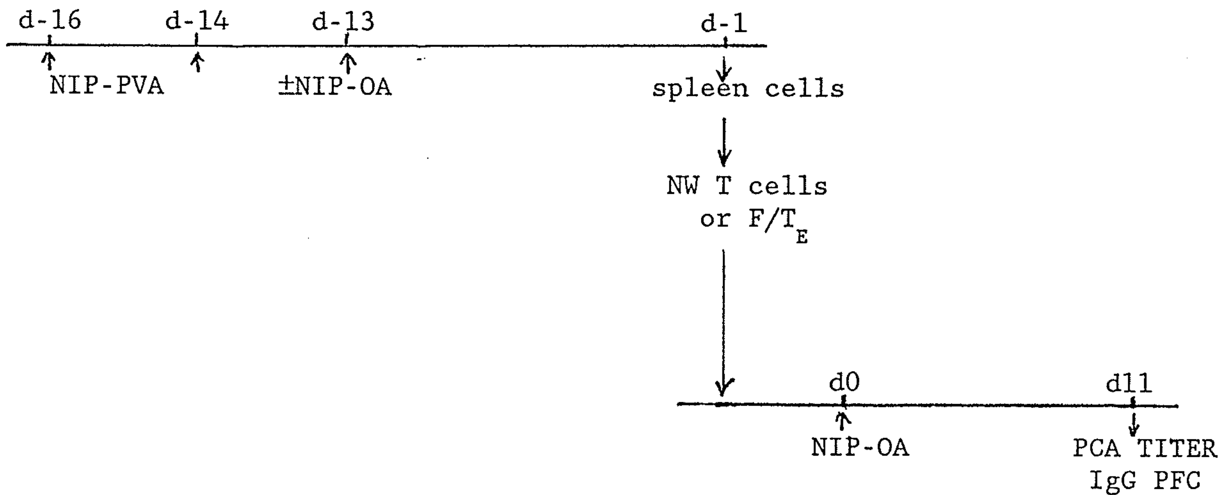


Table 13. Test for transfer of suppression after treatment of donors with NIP_4-PVA ± 1 μg NIP-OA+$Al(OH)_3$ (strain = C57BL/6 mice).

DONOR GROUP	TREATMENT OF DONORS ON DAY -16	-14	-13
1.	NIP-PVA	NIP-PVA	NIP-OA
2.	-	-	NIP-OA
3.	NIP-PVA	NIP-PVA	-
4.	-	-	-

	ANTI-NIP[1]		
	IgG PFC per 10^8 SC (d11)		PCA TITERS
RECIPIENTS ON D-1 OF NW T CELLS FROM DONOR GROUP #	IgM	IgG	(d11)
1.	8,200	14,100	850
2.	7,550	6,700	850
3.	8,750	3,250	<80
4.	7,100	13,200	800
-	13,900	51,300	3020
RECIPIENTS ON D-1 OF F/T$_E$ FROM DONOR GROUP #			
1.	2,200	10,100	400
2.	6,700	8,400	230
3.	10,900	6,200	115
4.	10,200	33,600	3470
-	9,000	27,000	3020

[1] 2 μg NIP-OA+$Al(OH)_3$ injected into recipients on d0

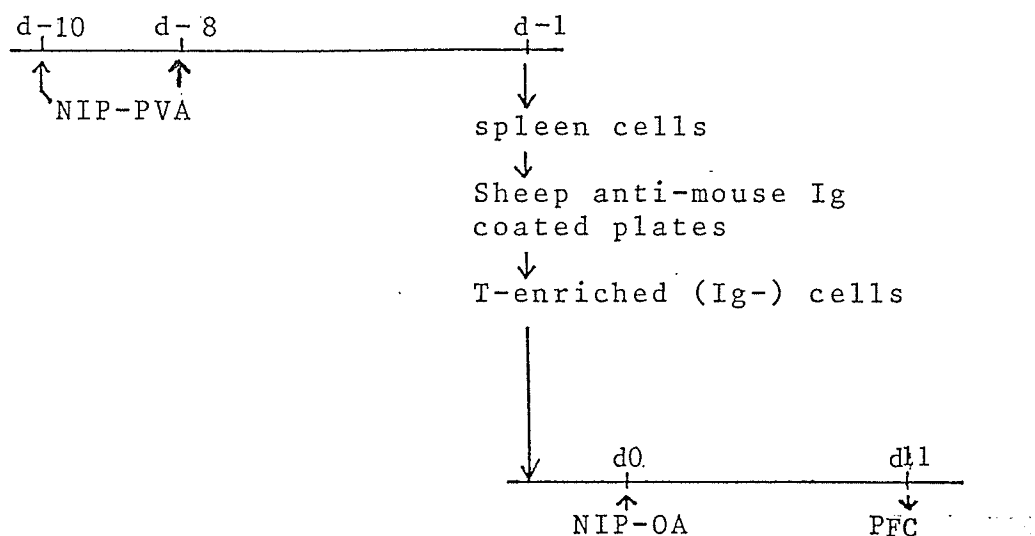


Table 14. Transfer of suppression by cells of C57BL/6 mice treated with NIP_4-PVA and by extracts of these cells.

TREATMENT OF DONORS	TREATMENT OF RECIPIENTS		anti-NIP PFC (d11) per 10^8 SC		PCA TITERS	
	d-1	d0	IgM	IgG	d9	d11
NIP-PVA (d-10,-8)	T_T	2 μg NIP_8-OA[1]	11,170	2,060	N.D.	N.D.
none	T_N	"	10,920	32,140	N.D.	N.D.
	none	"	12,380	37,860	N.D.	N.D.
NIP-PVA (d-15,-13)	F/T_T	"	N.D.	N.D.	210	110
none	F/T_N	"	N.D.	N.D.	1350	1350
	none	"	N.D.	N.D.	1280	1280

[1] in $Al(OH)_3$

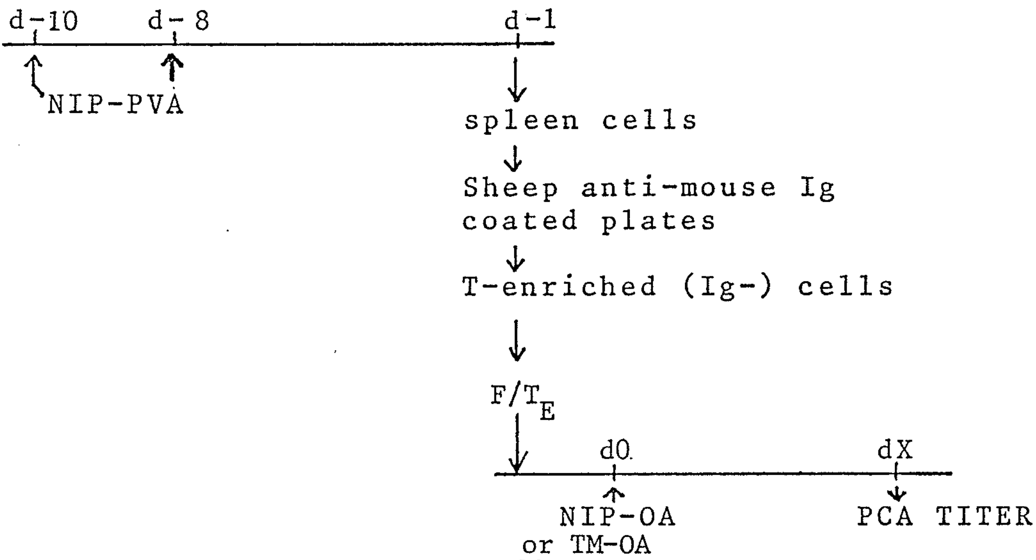


Table 15.a) Suppressive effect of F/T_E on a primary response to NIP_4-OA in C57BL/6 mice.

TREATMENT ON d-1 d0	PCA TITERS anti-NIP d11	d14	d21	anti-OA d11	d14	d21
1. F/T_T 3 μg	110	100	30	220	100	220
2. F/T_N NIP_4-OA[1]	825	1690	780	230	110	220
3. none	840	1690	240	420	115	110

b) Failure of the above F/T_E to suppress a primary IgE response to an unrelated hapten, TMA, presented on the same carrier, OA (strain = C57BL/6 mice).

TREATMENT ON d-1 d0	PCA TITERS anti-TMA d11	d14	d21	anti-OA d11	d14	d21
1. F/T_T 10 μg	1560	460	230	1730	1650	410
2. F/T_N TM_9-OA[1]	1650	780	390	1690	1730	825
3. none	1560	780	390	1650	1650	825

[1] in $Al(OH)_3$

munizing one set of recipients with TM_9-OA instead of NIP_4-OA, whereupon neither the anti-TMA nor the anti-OA responses were suppressed [Table 15.b)]. These results emphasized that the specificity of suppression transferred by the F/T_E was determined by the hapten coupled to the PVA with which donor mice had been treated. Therefore, the effect of NIP-PVA was to induce an NIP-specific suppressive mechanism, possibly involving NIP-specific suppressor T cells and suppressor factor(s).

3. Since the suppression was functionally hapten-specific, it seemed likely that NIP-specific suppressor factor(s) could be obtained from spleen T cells of NIP-PVA treated donors. An attempt was made to isolate hapten-specific suppressor factor(s) on hapten-coupled Sepharose 4B employing the F/T_E of spleen T cells from NIP-PVA treated donors. In this and all subsequent experiments, ShAMIg-coated plates were used to enrich for T (i.e. sIg-) cells. Preliminary experiments showed that neither NIP-BSA nor NIP-binding antibody bearing the NP^b idiotype, when coupled to Seph 4B, could remove the suppressive activity (data not shown). The running buffer (PBS) contained 0.01% C57BL/6 NMS which may have prevented binding due to the presence of natural antibodies against the NP^b id. Improvements were made on these experiments, as reported in Section III, b), 3.

4. Since the suppressive activity of the F/T_E could not be removed on an NIP-BSA-4B column and the amphipathic nature of PVA would favour binding to the spleen cells *in vivo* upon injection into mice, the possibility was entertained that NIP-PVA retained by the cell-free extracts of suppressive spleen T cells might be responsible

for the transfer of suppression. Naturally, the NIP-PVA would not bind to NIP-BSA-4B. Furthermore, such carry-over of NIP-PVA with the spleen T cells from NIP-PVA treated donors might be analogous to NIP being covalently coupled to spleen cells (50) for the purpose of providing a tolerogenic carrier for the NIP to induce a suppressor cell cascade.

III. Is the transfer of non-responsiveness due to the carry-over of NIP-PVA?

a) Anatomical distribution of NIP-PVA

Since carry-over of NIP-PVA was strongly considered among the possible mechanisms of transferable suppression, it was decided to establish the retention and distribution NIP-PVA following injection into C57BL/6 mice. An ^{125}I-labeled form of NIP-PVA i.e. N^{125}IP-PVA was synthesized in two steps, viz., NP-acetic acid was radio-iodinated to give N^{125}IP-acetic acid, which was then converted to N^{125}IP-*O*-succinimide for coupling to PVA (see MATERIALS AND METHODS). One mg of N^{125}IP-PVA was injected i.p. into mice on days -2 and 0 and then the animals were sacrificed on days 1,4 and 7 and the amount of radioactivity in various anatomic compartments was determined by counting in a gamma counter (Beckman, Gamma 300).

Tables 16 and 17 show the distribution of N^{125}IP-PVA in various organs on different days after injection. In all experiments, most of the N^{125}IP-PVA was deposited in the liver on all days tested up to day 7. The two kidneys had the next highest c.p.m., or about 5% of the c.p.m. in the liver. This would be expected, since the liver is known to trap foreign substances for detoxification or conversion be-

Table 16. Anatomical distribution of $N^{125}IP_4$-PVA at various times after injection into C57BL/6 mice.

ORGAN OR CONSTITUENT	% of original c.p.m. injected[1]					
	day 1		day 4		day 7	
	A[2]	B[3]	A	B	A	B
WHOLE MOUSE	45[4]	32	39	25	33	30
BLOOD (2.5 ml)	20	13.7	5.8	4.8	1.7	1.8
I. LIVER	17.3	11.4	16	10.2	10.9	11.2
SPLEEN	.34	.21	.41	.21	.43	.32
MESENTERIC LN	.27	.09	.20	.17	.30	.15
THYMUS + PTLN	.10	.05	.07	.05	.06	.06
KIDNEYS- R	.69	.38	.51	.29	.36	.28
- L	.63	.39	.46	.26	.34	.24
THYROID	N.D.	N.D.	N.D.	.30	N.D.	.24
URINE (per ml)	4.3	1.5	.53	.43	.46	N.D.
FAECES (per 100 mg)	.19	.07	.12	.11	.13	.04
II. MOUSE (remainder)	30	20.5	24	15.6	21.2	18.4
SUM OF I. + II.	49.3	33	41.7	27.1	33.6	30.9

[1] 1 mg NIP_4-PVA (i.p.) on d-2 and d0
[2] A= Experiment 1: total c.p.m. injected= 6.6×10^6
[3] B= Experiment 2: total c.p.m. injected= 7.0×10^6
[4] average of 2 mice for all values

...p. 73

...Table 16 (cont'd)

Measurement of c.p.m. in different fractions of the spleen 7d after injecting C57BL/6 mice with $N^{125}IP_{4}$-PVA (Expt. 2)[1]

Whole spleen cells	20,125 c.p.m. /3.55×10^{8} SC or 2.3 μg NIP-PVA/ 10^{8} WSC.
Spleen T (non-adherent)[2]	740 c.p.m./ 3.2×10^{7} cells or 186 ng NIP-PVA/ 2×10^{7} T cells

[1]SC purified on Ficoll-metrizoate
[2]sIg+ cells were removed on Shanti-MIg-coated plates

After F/T of spleen T cells:[1]

FRACTION	C.P.M.[2]	ng
Supernatant	246	74 ng NIP-PVA/ 2×10^{7} cell equiv.
Pellet	413	
	659	

[1]2.8×10^{7} cells were suspended in 1.9 ml and subjected to F/T as described in MATERIALS AND METHODS.
[2]c.p.m. above background (160 c.p.m.)

Table 17. Anatomical distribution of $N^{125}IP_3$-PVA 7 days after injection into C57BL/6 mice.

ORGAN OR CONSTITUENT	% of original c.p.m. injected[1] MOUSE: 1	2	3	AVERAGE
WHOLE MOUSE	28.0	26.8	26.5	27.1
LIVER	13.5	12.7	12.7	13.0
MESENTERIC LN	.10	.12	.04	.09
THYMUS	.03	.05	.03	.04
KIDNEYS- R	.34	.33	.32	.33
- L	.33	.29	.31	.31
THYROID	N.D	N.D	.32	.32

ORGAN MOUSE:	1	2	3	4	5	AVERAGE
SPLEEN	.22	.28	.21	.28	.23	.24

[1] 7.2×10^6 c.p.m. total (1 mg NIP_3-PVA i.p. on d-2 and d0)

Measurement of c.p.m. in different fractions of the spleen 7d

Spleen T (non-adherent)[1]	581 c.p.m./ 1.17×10^8 cells
	or 27 ng NIP-PVA/ 2×10^7 T cells
Spleen B (adherent)	660 c.p.m./ 9×10^7 cells
	or 40 ng NIP-PVA/ 2×10^7 B cells

After F/T of spleen T cells:[2]

FRACTION	C.P.M.[3]	ng
Supernatant	197	11.2 ng NIP-PVA/ 2×10^7 cell equiv.
Pellet	199	
	396	

[1] WSC were isolated on Shanti-MIg-coated plates
[2] The sup't had 9.5×10^7 cell equiv. in 0.95 ml,
[3] c.p.m. above background (100 c.p.m.)

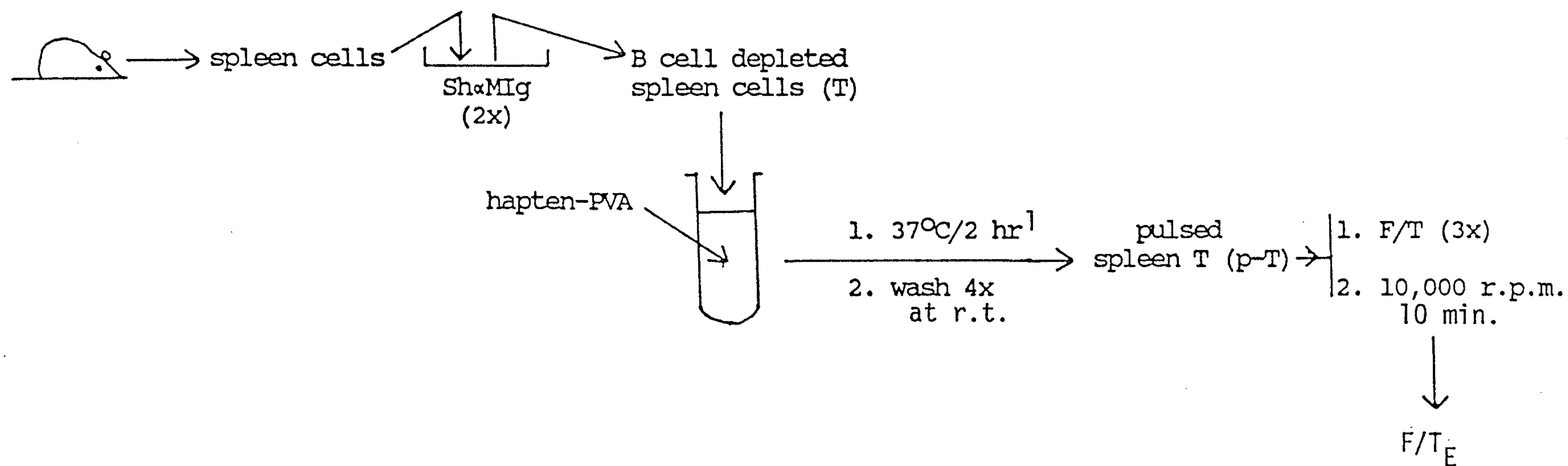
fore elimination through the kidneys. Of all lymphoid organs, the spleen had the most residual $N^{125}IP$-PVA, which could simply be a result of the relative size of this organ. Finally, washed spleen T cells isolated on day 7 were also examined in order to determine the amount of $N^{125}IP$-PVA that might have been carried over with these cells at the time of cell transfer in previous experiments. The amount of $N^{125}IP$-PVA contained in the F/T_E of these spleen T cells was also determined.

The combined data in Tables 16 and 17 demonstrated that when $N^{125}IP$-PVA was injected into mice, using the normal procedure for obtaining transfer of non-responsiveness, $2x10^7$ spleen T cells still retained from 27 to 186 ng of the injected NIP-PVA. In addition, freezing and thawing of these T cells liberated from 11 to 74 ng of the retained NIP-PVA supernatant. Therefore, in previous experiments using the same injection schedule for NIP-PVA, these amounts of NIP-PVA had been transferred. It remained to be determined whether the presence of NIP-PVA in the extracts of the spleen T cells was the reason for the transfer of non-responsiveness.

A method had to be devised to artificially put a known amount of NIP-PVA onto normal splenic T cells in order to avoid having any possible suppressor T cells induced and to determine the lowest amount of NIP-PVA which could still transfer non-responsiveness to recipient mice. Therefore, an *in vitro* model for the association of NIP-PVA and spleen T cells was established, mimicking the type of binding (specific and/or non-specific) which would allow NIP-PVA to become attached to spleen T cells *in vivo*. The *in vitro* model did not allow for suppressor cell induction, since the time of pulsing

was only 2 hrs. Normal T cells were pulsed and washed (see Fig. 6 for method) under conditions such that their F/T_E contained an amount of NIP-PVA similar to that reported in Table 16. Under the pulsing conditions described in Fig. 6, there was a close correlation between the concentration of $N^{125}IP$-PVA used for pulsing (x-values for Figs. 7 and 8) and the nanograms of NIP-PVA retained on the spleen T cells or in the F/T_E (y-values for the same Figs.) with the correlation coefficient r=0.94 for Fig. 7 and r=0.97 for Fig. 8 (3 separate expts. for each). This implied as the concentration of NIP-PVA in the pulsing medium and presumably in the spleens of *in vivo* treated mice increased, so did the amount of NIP-PVA which remained attached to the spleen T cells after washing. The final conditions chosen for future experiments were 2 mg of NIP-PVA incubated with $1x10^8$ spleen T cells in 2 ml of culture medium for a final amount in the F/T_E of 79 ng/ $2x10^7$ cell equiv. This amount of NIP-PVA was just above the range previously ascertained for NIP-PVA on spleen T cells of NIP-PVA-treated mice (11-74 ng/ $2x10^7$ cell equiv.).

FIGURE 6. Spleen T cells pulsed *in vitro* with N^{125}IP-PVA.



[1]Conditions: 2 mg N^{125}IP-PVA/10^8 spleen T cells
in 2 ml RPMI + 3% FCS + $NaHCO_3$
in 5% CO_2 atmosphere

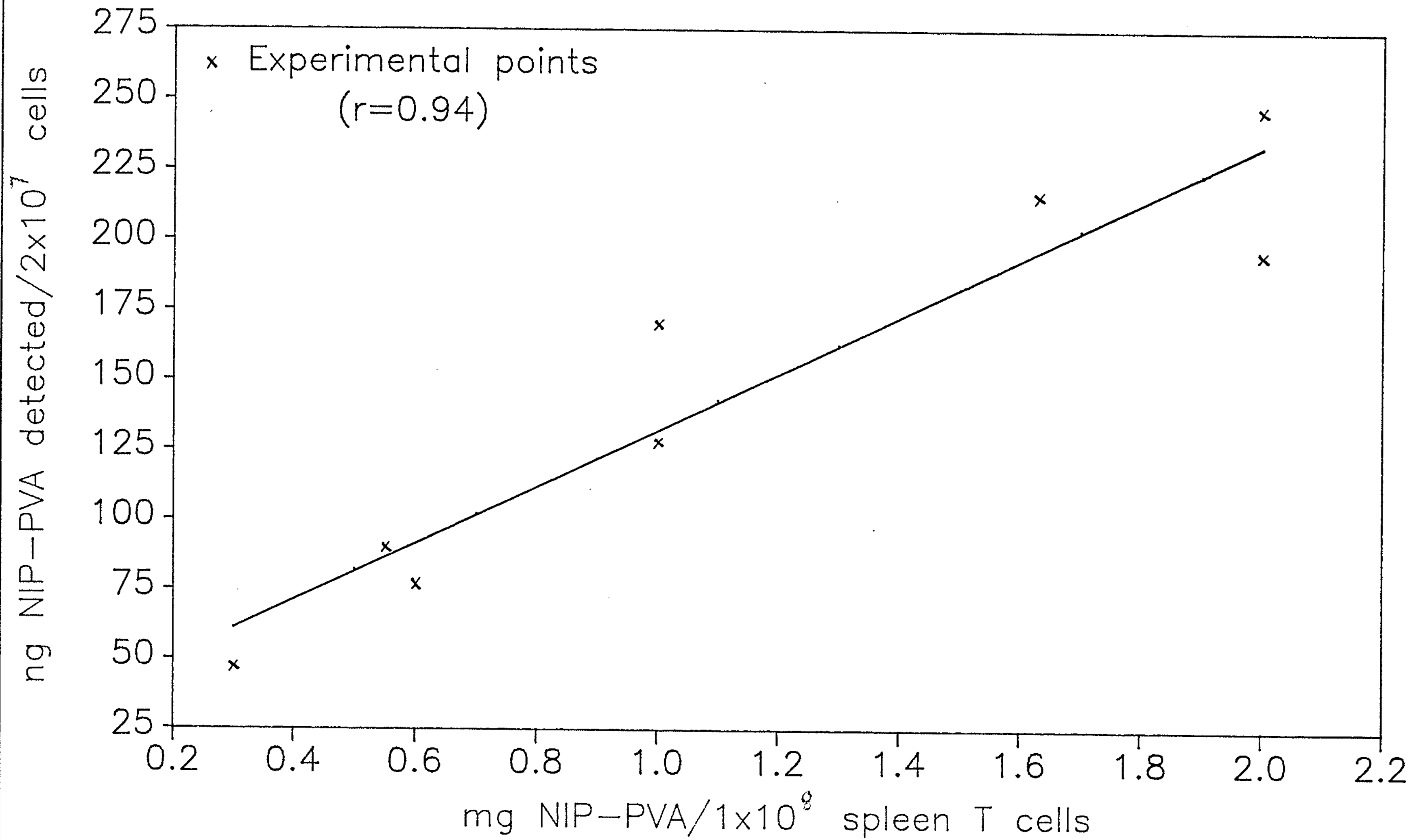


FIGURE 7
NIP-PVA¹ RETAINED AFTER PULSING
SPLEEN T CELLS
× Experimental points
(r=0.94)
ng NIP-PVA detected/2x10⁷ cells
mg NIP-PVA/1x10⁹ spleen T cells
275
250
225
200
175
150
125
100
75
50
25
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
1 N(125)IP-PVA

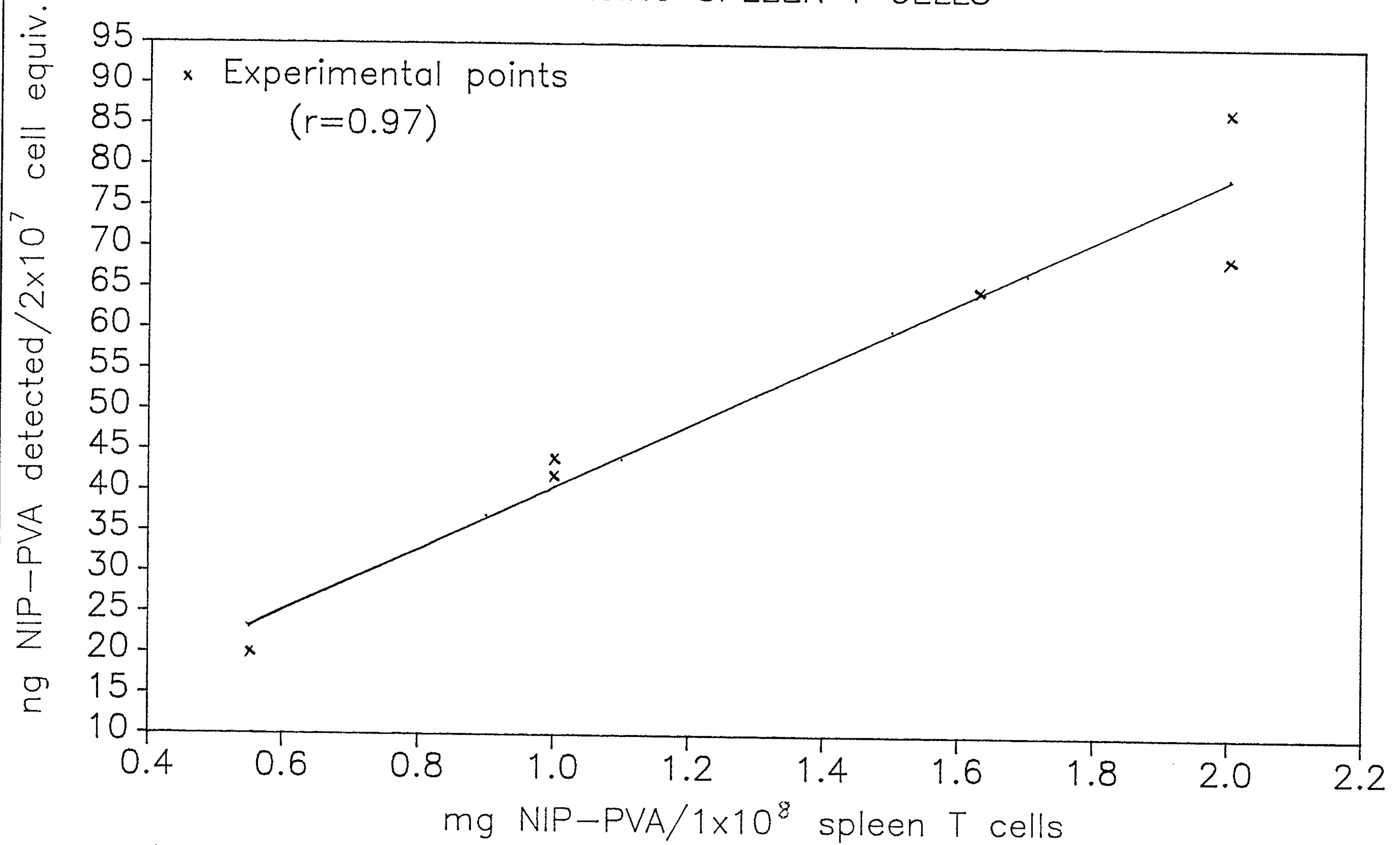


FIGURE 8
NIP−PVA¹ RETAINED ON F/T AFTER
PULSING SPLEEN T CELLS
× Experimental points
(r=0.97)
ng NIP−PVA detected/2x10⁷ cell equiv.
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
mg NIP−PVA/1x10⁸ spleen T cells
1 N(125)IP−PVA

b) <u>Evidence of carry-over as a possible mechanism of suppression</u>

1. The fact that the NIP-PVA could be pulsed onto normal spleen T cells to imitate the association of NIP-PVA with spleen T cells from NIP-PVA treated mice, made possible a comparison between the suppressive effects of the F/T_E of pulsed spleen T cells (*in vitro* F/T_E) and the effects of the F/T_E of spleen T cells from NIP-PVA treated donors (referred to as *in vivo* F/T_E). Doses of the F/T_E representing between 1 and 125 ng of NIP-PVA were injected into naive recipients one day before the recipients were immunized with NIP-OA. As shown in Table 18, it was possible to suppress significantly the IgE responses of mice to NIP-OA by injecting the *in vitro* F/T_E (Expts. A, B, C and D). As low as 10 ng of NIP-PVA released into the F/T_E could suppress the IgE response to NIP by 80-90%.

Since free NIP-PVA might have been released from the F/T_E of pulsed spleen T cells, it was also necessary to determine whether free NIP-PVA could suppress at the ng amounts released into the F/T_E. Table 18 has several experiments (Expt. A, B, C and E) in which the free NIP-PVA had as strong an effect at 10-100 ng free as the *in vitro* F/T_E, when given one day before immunization. This suggested that the NIP-PVA carried over in the amounts of 10-100 ng/ $2x10^7$ spleen T cells could be released upon freezing and thawing into the supernatant as free NIP-PVA.

2. If carried-over NIP-PVA was the suppressive moiety in the F/T_E, then it was expected that the suppressive activity would be absorbed out by anti-NP-4B. To confirm that the failure to bind the suppressive activity in F/T_E to either NIP-BSA-4B or B6anti-NP-4B (see II, 3.) immunosorbent columns was not simply due to technical problems,

Table 18. Test of effective dose range for NIP-PVA in two different states: 1) released into F/T_E of in vitro-pulsed spleen T cells; 2) in free solution.

EXPT.		HAPTEN COUPLED TO PVA	STATE OF HAPTEN-PVA	AMOUNT OF NIP-PVA (ng)	anti-NIP		anti-OA
					d13		d13
A.	1.	NIP	F/T_E	80	110		110
	2.	NIP	"	40	220		220
	3.	NIP	"	20	60		110
	4.	DNP	"	80	840		230
	5.	NIP	free	80	<80		<40
	6.	-	-	-	440		220
					d10	d14	
B.	1.	NIP	F/T_E	125	30	30	
	2.	NIP	"	62	25	30	
	3.	NIP	"	12	40	50	
	4.	NIP	"	1.2	75	100	
	5.	DNP	"	100	220	400	
	6.	NIP	free	10,000	610	215	
	7.	NIP	"	1,000	170	115	
	8.	NIP	"	100	60	60	
	9.	NIP	"	10	155	50	
	10.	-	-	-	420	150	
	11.	-	-	-	1140 / 690 [1]	390 / 240 [1]	
							d14
C.	1.	NIP	F/T_E	65	50	100	30
	2.	NIP	"	10	195	190	
	3.	NIP	"	1	810	860	
	4.	DNP	"	65	460	1440	360
	5.	NIP	free	10,000	420	420	195
	6.	NIP	"	1,000			
	7.	NIP	"	100	<80	210	195
	8.	NIP	"	10	210	430	
	9.	-	-	-	1610	1560	360
	10.	-	-	-	1690 / 1650 [1]	900 / 1185 [1]	420 / 390 [1]
D.	1.	NIP	F/T_E	63	230	220	
	2.	NIP	"	32	110	210	
	3.	NIP	"	16	210	330	
	4.	DNP	"	53	1560	1730	
	5.	-	-	-	880	1560	
E.	1.	NIP	free	10,000	<80	390	
	2.	NIP	"	100	60	110	
	3.	NIP	"	10	55	100	
	4.	-	-	-	825	720	
	5.	-	-	-	865 / 845 [1]	420 / 550 [1]	

[1] Geometric mean of titres for controls receiving only NIP-OA + $Al(OH)_3$ on d0

the experiments were repeated with new conditions that showed specific binding of N^{125}IP-PVA to the anti-NP- 4B (see MATERIALS AND METHODS). The method involved a batchwise incubation of the sample to be absorbed with the immunoadsorbent gel, and elution of the unbound and bound fractions of the sample in a column. Thus, the F/T_E and immunosorbent were stirred continuously in suspension without NMS, rather than the F/T_E being applied to a column of immunosorbent in the presence of 0.01% NMS as before.

The binding of N^{125}IP-PVA to the anti-NP-4B was also measured in the presence of the biological components of the F/T_E. N^{125}IP-PVA (3.2 x10^6 c.p.m./ mg) was injected into mice, and 7d later the F/T_E from the SC was passed through XM-300 (see Table 27), retained on XM-100A, then added (as 200-300 c.p.m.) to each of B6anti-NP and B6nIg-Seph 4B. The % bound for anti-NP vs. nIg was 68% (98-30). Since the presence of the F/T_E substantially reduced non-specific binding of N^{125}IP-PVA to the B6anti-NP-4B (see the binding of N^{125}IP-PVA alone in MATERIALS AND METHODS), 3x10^7 equivalents of F/T_E from normal spleen T in 0.5 ml MEM with 700-1,000 c.p.m. (N^{125}IP-PVA) was added to either gel. The specific % bound was 87% (94-7). An attempt was made at this point to elute the N^{125}IP-PVA with glycine-HCl, pH 3.2:

Fraction	c.p.m.	%
B6anti-NP (after Gly-HCl)	644	85
PBS wash (2.0 ml)	0	
Gly-HCl eluate (2.0 ml)	114	15
	758	

Clearly, elution with glycine-HCl had not removed the N^{125}IP-PVA

bound to the B6anti-NP column; this was to provide an explanation for the later finding that the suppressive activity which was lost from the effluent could not be completely recovered in the eluant (Table 20, d10). The B6anti-NP gel was tested once more to confirm the binding of $N^{125}IP$-PVA (Table 19). It was shown that guanidine-HCl, but not glycine-HCl could elute all of the bound $N^{125}IP$-PVA.

The implication from all these results was that if suppressive activity in the F/T_E was due to residual NIP-PVA, the suppressive activity should also bind to the B6anti-NP column. In order to improve binding conditions, the initial incubation of F/T_E ($2x10^8$ cell equivalents in 2 ml) with an equal volume of immunoadsorbent was also performed using a batch procedure. Furthermore, the F/T_E also contained PMSF (50 μg/ml in the MEM or HBSS of the original cells) to inhibit the breakdown of any suppressive factor(s) by proteases. The aim was to allow for optimal interaction of coupled ligand and the suppressive entity in the extract during the gentle mixing over 1 hr. The removal of any unbound material was again gently performed by washing the gel with PBS (no NMS) in the same tube, removing the supernatant carefully after each centrifugation.

The final elution of bound material was performed with the same gel (2 ml) packed into a small Bio-rad column with a nylon net at the bottom. The eluant was 5.6 ml 0.2M Glycine-HCl, pH 3.2 and elution was into a tube containing 0.1 ml 2M Tris-HCl, pH 8.0 and 0.63 ml of F/T_E (nor). The final pH of the eluate was 7.2-7.3. Mice were injected with the effluent and eluate fractions in 1 ml containing $3x10^7$ equivalents of spleen T cells (Table 20).

The same procedure was followed with the NIP-BSA-Seph4B, to con-

Table 19. Binding of $N^{125}IP$-PVA to B6anti-NP-Seph 4B but not to the control immunoadsorbent, B6nIg-Seph 4B, in the presence of F/T_N.

4B-COLUMN	FRACTION	C.P.M.	% OF TOTAL		
B6anti-NP (for sample adsorption)	effluent	44	3.6	11.2	
	PBS wash 1	55	4.6		
	" 2	23	1.9		
	" 3	13	1.1		
	gel-bound	1071	88.8		
	total	1206			
B6anti-NP (for sample elution)	PBS wash (2 ml)	4	0	20.1	96.4
	Gly-HCl (1.4 ml)	95	11.5		
	" (1.4 ml)	71	8.6		
	Guan.-HCl (2 ml)	632	76.3		
	PBS (3 ml)	0	0		
	gel-bound	30	3.6		
	total	832			
B6nIg(M+G) (control immunoadsorbent)	effluent	535	47.3	95.1	
	PBS wash 1	350	30.9		
	" 2	127	11.2		
	" 3	65	5.7		
	gel-bound	54	4.8		
	total	1131			

Table 20. Ability of B6anti-NP-Seph 4B to absorb out suppressive activity of F/T_E.

EXPT.	TREATMENT OF DONORS	FRACTION[1] INJECTED INTO RECIPIENTS	ANTI-NIP[2] PCA TITERS d10	d14	d17
A.	1. NIP-PVA	effluent[3]	430	750	430
	2. $DNP_{1.45}$-PVA	effluent[3]	780	825	410
	3. NIP-PVA	eluate[3]	450	200	110
	4. $DNP_{1.45}$-PVA	eluate[3]	825	780	235
	5. -	-	640	1560	865
B.	1. NIP-PVA	effluent[4]	390	220	
	2. $DNP_{1.45}$-PVA	effluent[4]	780	240	
	3. NIP-PVA	eluate[4]	205	60	
	4. $DNP_{1.45}$-PVA	eluate[4]	450	230	
	5. -	-	430	240	

[1]Fractions from B6anti-NP-Seph 4B gel (see MATERIALS AND METHODS)
[2]Recipients immunized with 3 μg NIP_4-OA+$Al(OH)_3$ on d0
[3]$3x10^7$ cell equiv. of F/T_E injected on d-1
[4]$3.5X10^7$ cell equiv. of F/T_E injected on d-1

firm the previous findings that the suppressive activity was not NIP-binding. It was found that the suppressive extracts again did not bind to the NIP-BSA-4B (Table 21). It should be noted that the eluates contained some non-specific suppressive activity, regardless of the source of the F/T_E (Groups 3 and 4). In contrast, with a freshly prepared B6anti-NP-4B gel, 2 experiments indicated that the suppressive moiety could indeed be absorbed out by the reverse immunoadsorbent (Table 20, Group 1) and at least partially recovered in the eluate (Group 3). Since it was shown previously that only 15-20% of gel-bound NIP-PVA could be eluted with glycine-HCl, it was not surprising that all the suppressive activity could not be recovered in the eluate (Group 3, d10). This result could be explained if one assumed that carried-over NIP-PVA was the sole entity transferring the suppression.

3. If NIP-PVA induced T_s cells, a longer time than 2 days would probably be required. If, however, carry-over was a significant factor in the transfer of suppression, it could be expected that the F/T_E of spleen T cells removed shortly after initial NIP-PVA treatment should be able to suppress the NIP-specific IgE response. Indeed, the F/T_E transferred suppression only 2 days after treatment with NIP-PVA (Table 22). This early transfer of suppression would not likely be due to activation of suppressor cells, which usually requires between 4-7 days (see LITERATURE REVIEW).

4. Exploratory experiments indicated that the cells carrying NIP-PVA were able to transfer suppression despite treatment with anti-Thyl.2 + C′ [Table 23.a)] or irradiation [Table 23.b)], which should have abolished suppressor cell activity. This reinforced the

Table 21. Failure of F/T_E to bind to NIP-BSA-Seph 4B.

EXPT.		TREATMENT OF DONORS	FRACTION[1] INJECTED INTO RECIPIENTS	ANTI-NIP[2] PCA TITERS d10	d14
A.	1.	NIP-PVA	effluent[3]	55	55
	2.	$DNP_{1.45}$-PVA	effluent[3]	640	825
	3.	NIP-PVA	eluate[3]	206	105
	4.	$DNP_{1.45}$-PVA	eluate[3]	410	360
	5.	-	-	780	1560
	6.	-	-	640	1730
				705[4]	1640[4]
				d11	d14
B.	1.	NIP-PVA	effluent[3]	220	215
	2.	$DNP_{1.45}$-PVA	effluent[3]	840	1440
	3.	NIP-PVA	eluate[3]	115	195
	4.	$DNP_{1.45}$-PVA	eluate[3]	450	205
	5.	-	-	1440	1440
	6.	-	-	805	420
				1075[4]	780[4]

[1] Fractions from B6anti-NP-Seph 4B gel (see MATERIALS AND METHODS)
[2] Recipients immunized with 3 μg NIP_4-OA+$Al(OH)_3$ on d0
[3] $3x10^7$ cell equiv. of F/T_E injected on d-1
[4] Geometric mean of titers for controls receiving only NIP-OA

Table 22. Transfer of suppression 2d or 3d after initial NIP_4-PVA treatment (strain = C57BL/6 mice).

EXPT.	NIP-PVA TO DONORS ON DAYS	SPLEEN T CELLS ISOLATED ON DAY	ANTI-NIP[1] PCA TITERS (d11) TREATMENT OF RECIPIENTS ON D-1		
			F/T_T	F/T_N	-
A.	-3 + -1	0	480	3020	1700
	"	6	60 (60)[2]	1410 (205)	1620 (230)
B.	-2 + -1	0	110	1490	400
	"	6	190	760	1410

[1] recipients immunized with 2 μg NIP_8-OA+$Al(OH)_3$ on d0
[2] anti-OA PCA titers

Table 23.a) The transfer of suppression by whole spleen cells from NIP_4-PVA treated donors[1] is not sensitive to treatment with anti-Thy1.2 + C'

	DONORS	TREATMENT OF WHOLE SPLEEN CELLS	PCA TITERS anti-NIP[2] d13	PCA TITERS anti-NIP[2] d20
1.	Tolerized	anti-Thy1.2 + C'[3]	<160	<20
2.	"	C'	120	<20
3.	Normal	C'	760	210
4.	none	none	1480	420

[1] treatment of C57BL/6 mice on d-15
[2] recipients immunized with 2 μg NIP_8-OA+$Al(OH)_3$ on d0
[3] 25% T cell-specific lysis of SC

Table 23.b) Resistance of transfer[1] of suppression to irradiation (650 R before injection into C57BL/6 mice).

	RECIPIENTS d-1	RECIPIENTS d0	650 rads to T cells	PCA TITERS (d14) anti-NIP	PCA TITERS (d14) anti-OA
1.	T_T	3 μg NIP_4-OA[2]	+	30	105
2.	T_T	"	-	10	20
3.	T_N	"	-	150	30
4.	none	"		1690	435

[1] 7d after treatment of donors with NIP_4-PVA
[2] in $Al(OH)_3$

view that suppressor cells were not necessary to account for the transfer of suppression in the NIP-PVA system.

5. The association of NIP-PVA with spleen T cells or other cells in the spleen could occur through the cell membrane, since NIP-PVA is expected from its structure to have amphipathic properties (with hydrophobic regions contributed by the vinyl groups and the hydroxy groups affording hydrophilicity). The injection of spleen T cell extracts containing carried-over NIP-PVA might suppress the recipients by presenting hapten-modified self to a Ts_1 cell, as in the system with NP-modified syngeneic cells (32a). However, since no genetic restrictions have been found for the activity of such NIP-PVA associated with cell extracts (Table 24), this lends weight to the argument that free NIP-PVA is the agent responsible, although it is still possible that host macrophages would be able to process hapten coupled to allogeneic membranes in order to present the foreign membranes in the context of modified self (16). In one experiment (Table 25) $2x10^6$ splenic adherent cells pulsed with the F/T_E of spleen T cells from NIP-PVA-treated donors could transfer suppression for the IgE response. This result supported the idea that NIP-PVA in the F/T_E could be presented as modified self by macrophages (splenic adherent cells) in the recipients, but simple carry-over of the NIP-PVA at a low dose would also explain this result.

IV. Further characterization of free and cell-derived NIP-PVA

1.a) M.W. characterization on XM-100A and XM-300 ultrafiltration membranes showed that NIP-PVA from the same preparation either 1) free in solution (Table 26) or 2) from *in vivo* or *in vitro* F/T_E

Table 24. Suppressive activity of F/T_E from C57BL/6 mice is not linked to either the MHC[1] or the Igh-V[1] region.

Recipient Strain	Day	ANTI-NIP[2] PCA TITERS F/T_E (tol)	F/T_E (nl)	No Treatment	% Suppression[3]
B/6	d11	<20	870	1230	>97
	d15	<40	200	440	>80
B10.BR	d11	<10	55	320	>81
	d15	14	95	760	85
C3H	d11	55	710	440	92
	d15	30	390	320	92
C3H.SW	d11	<40	380	380	>89
	d15	100	110	230	-
B/6	d13	55	1410	5750	96
	d22	50	110	410	54
BALB/c	d13	<20	1490	730	>98
	d22	<20	760	810	>97

[1]Haplotypes of mice at H-2 and Igh-V genes

	H-2	Igh-V
C57BL/6	b	b
B10.BR	k	b
C3H	k	j
C3H.SW	b	j
BALB/c	d	a

[2]recipients immunized with 2 μg NIP_8-OA+$Al(OH)_3$ on d0, except for BALB/c (10 μg)
[3]% suppression= 1-(tol/nl)

Table 25. Transfer of suppression by splenic adherent cells pulsed with F/T_E.

TREATMENT OF RECIPIENTS[1]	ANTI-NIP PFC/10^8 WSC (d11) IgM	IgG	PCA TITERS (d11) IgE
1. SAC pulsed with F/T (tol)	14,400	12,800	340
2. SAC pulsed with F/T (nl)	15,610	21,760	1490
3. No treatment	12,500	29,250	2560

[1]$2x10^6$ cells per recipient for Gps. 1 and 2, 1d before immunization with 2 μg NIP_8-OA+$Al(OH)_3$ on d0

Table 26.a) Behaviour of $N^{125}IP$-PVA from different sources on ultrafiltration membranes.

Form of $N^{125}IP$-PVA		Amount in Filtrate	Amount in Retentate	Amount Adherent to Filter	Total c.p.m.
F/T_E (*in vivo*)	%	4	82	10	4,175
	ng	-	--	140	
$N^{125}IP$-PVA	%	8	3	94	112,000
	ng	-	-	35,000	
$N^{125}IP$-PVA + 10 mg cold NIP-PVA					
Expt. 1	%	33	54	8	112,000
	μg	-	-	800	
Expt. 2	%	38	38	3	92,000
	μg	-	-	300	

This data showed that an average of 500 μg of NIP-PVA could bind non-specifically to the XM-100A filter, thereby suggesting that the protein contained in the F/T_E was somehow preventing this binding.

Table 26.b) Fractionation of $N^{125}IP$-PVA on ultrafiltration membranes.

XM-300	FRACTION	Total c.p.m.	% of original
Filter precoated with spl T F/T (nl)	Original	55,858	
	Retentate	22,335	40
	Filtrate	20,822	37
	Filter	1,934	3
Filter not precoated	Original	55,613	
	Retentate	511	0
	Filtrate	14,422	26
	Filter	38,737	70
Filters precoated with SC_E (nl)			
XM-300:	Original	20,275	
	Retentate	2,203	11
	Filtrate	13,342	66
	Filter	651	3
XM-100A:	Original	23,063	
	Retentate	8,330	36
	Filtrate	5,950	26
	Filter	3,970	17

[Table 26.a) and 27] could pass through XM-300 and was partially retained on XM-100A, implying a common molecular weight of about 120 kD.

b) The suppressive activity of the F/T_E of *in vivo* treated spleen T cells was retained by XM-100A (Table 28), indicating a molecular size >120 kD. This result contrasts with findings in laboratories which have reported molecular weights <70 kD for monomeric suppressor factors.

c) An *in vivo* F/T_E was passed through an ACA-44 gel filtration column to determine the eluting volume of the suppressive activity (see Fig. 9). It was discovered that fraction 1 corresponding to a M.W. >95 kD contained all the suppressive activity compared to the control response of mice receiving the F/T_E of normal T cells (Table 29). This fraction could have contained free NIP-PVA, since Fig. 10 shows that free NIP-PVA (M.W. 14,000) eluted at the same fraction of the ACA-44 column due to its elongated structure, rather than at the position expected for a globular protein of the same M.W. The same discordance between the M.W. of NIP-PVA and the expected behaviour of a protein with that M.W. was noted for the ultrafiltration membranes. The ACA-44 column fractionation confirms that free NIP-PVA carried over in the F/T_E could be directly responsible for the suppressive effect, and this could explain the absence of evidence for a protein-like suppressor factor inhibiting NIP-specific responses after NIP-PVA treatment.

Table 27. Behaviour of different forms of $N^{125}IP$-PVA on ultrafiltration membranes.

Filter and sample	Fraction after Filtration	Total c.p.m.	% of original
XM-300: S.F. (*in vivo*)	Retentate	74	5
	Filtrate	987	71
	Filter	102	7
XM100A: Filtrate of XM-300 above	Retentate	455[1]	61
	Filtrate	116	16
	Filter	150	20
XM-300: $N^{125}IP$-PVA	Retentate	408	0
	Filtrate	4,272	8
	Filter	38,411	74
XM-100A: Spl T F/T_E (*in vitro*)	Retentate	164	30
	Filtrate	182	34
	Filter	124	23
XM-300: Retentate + Filtrate of XM-100A above	Retentate	29	8
	Filtrate	330	95
	Filter	67	19

[1]This sample was further studied in terms of its ability to bind to B6anti-NP-4B (see pg. 82).

Table 28. Retention of suppressive activity of F/T_E on XM-100A ultrafiltration membrane.

	TREATMENT OF RECIPIENTS		PCA TITERS (d13)	
	d-1	d0	anti-NIP	anti-OA
F/T(tol)	Filtrate	NIP_4-OA (3 μg)[1]	1620	N.D.
	Retentate	"	<80	<20
F/T (nl)	Filtrate	"	890	N.D.
	Retentate	"	440	120
	--	"	810	120

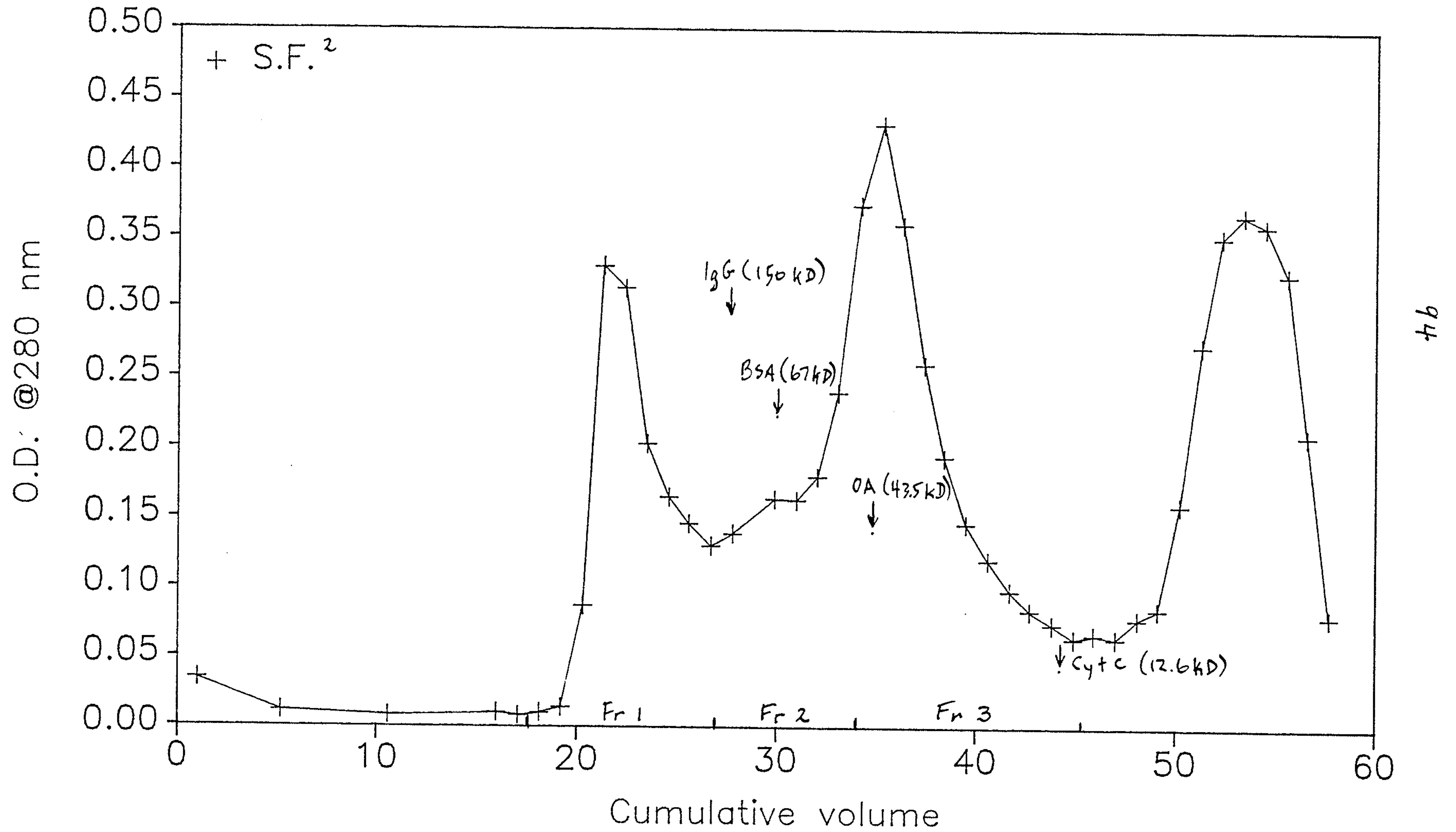
[1]in $Al(OH)_3$

Table 29. Isolation of soluble suppressive extract on ACA-44 column.

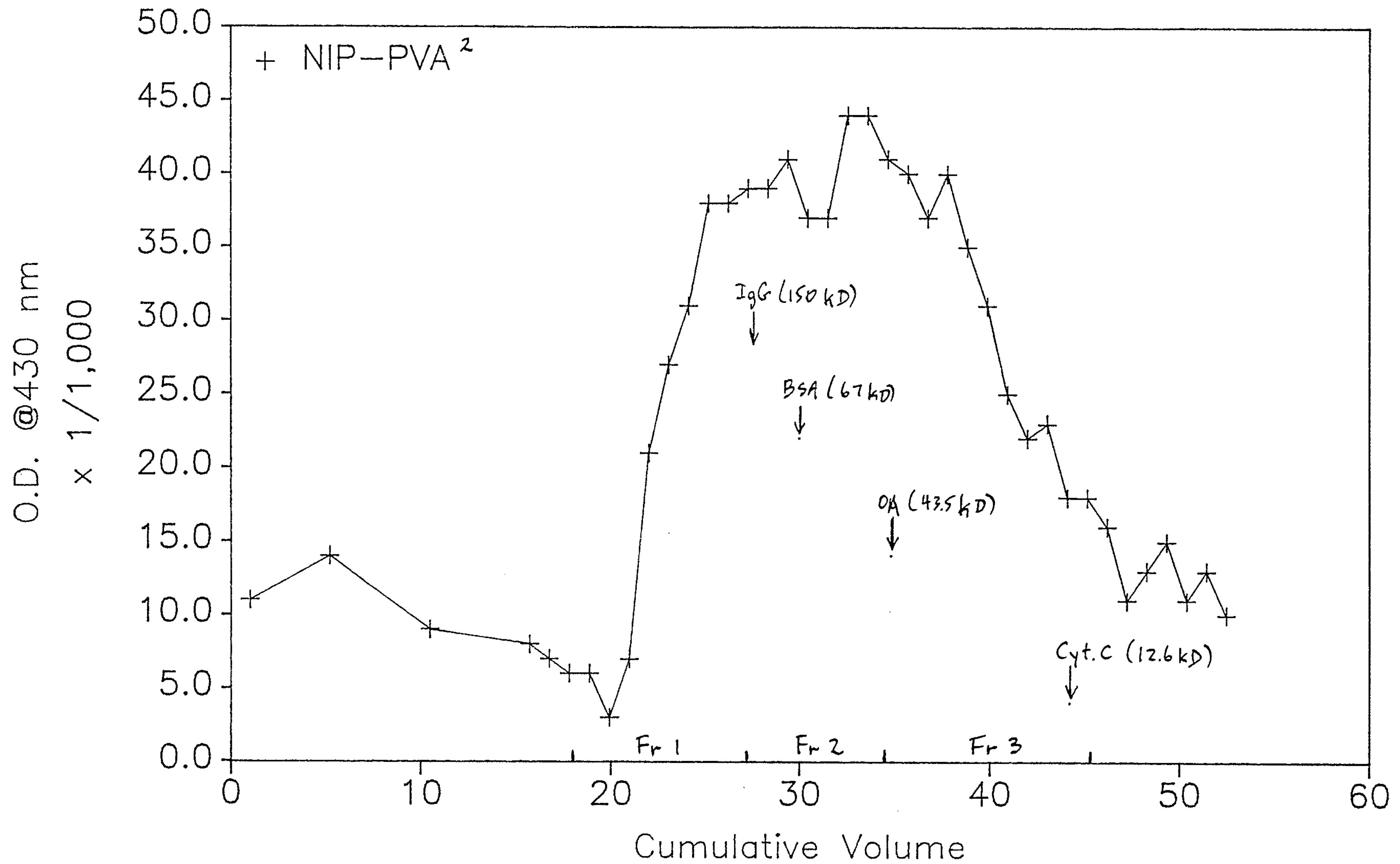
FRACTION INJECTED[2] ON DAY -1	$PFC/10^8$ WSC (d11) IgM	IgG	ANTI-NIP[1] PCA TITERS (d11)	M.W. OF FRACTION
1. F/T (tol)- unfrac.	15,015	4,640	<20	-
2. F/T (nl) - unfrac.	15,870	10,750	100	-
3. F/T (tol)- Fraction 1	27,930	16,760	<20	>95KD
4. " - " 2	17,370	17,850	180	34-95KD
5. " - " 3	15,680	15,010	230	10-34KD
6. No treatment	22,750	46,250	440	

[1]recipients immunized with 2 μg NIP_8-OA+$Al(OH)_3$ on d0
[2]$2x10^7$ cell equiv. for Gps. 1 and 2; $7.5x10^7$ cell equiv. for Gps. 3-5

FIGURE 9
FRACTIONATION OF SUPPRESSIVE EXTRACT
ON ACA-44 COLUMN[1]



1 void volume= 22 ml
2 3.1×10^8 cell equivalents in 1 ml



1 void volume= 22 ml
2 0.5 mg NIP–PVA in 0.5 ml

V. Effect of cyclophosphamide on the suppression by low doses of NIP-PVA

Since low doses of NIP-PVA could down-regulate NIP-specific IgE responses, It was of interest to know what mechanism of suppression existed in these recipients. Cy treatment was one method to distinguish between direct tolerance and induction of suppressor cells, due to the particular sensitivity of suppressor cell presursors to Cy (76).

Either with 100 ng of free NIP-PVA (Table 30, A.) or 50 ng NIP-PVA supplemented with $2X10^7$ spleen cell equivalents of F/T_E to imitate the usual *in vivo* F/T_E (Table 30, B.) the IgE response was suppressed. 100 mg/kg cyclophosphamide (Cy) given i.p. 3d before treatment with these low doses of NIP-PVA could counteract the suppression (Table 30, A. and B.). Therefore, suppressor cells were putatively revealed as one mechanism of suppression by low doses of NIP-PVA. It was also shown that either i.v. or i.p. injection of 100 ng NIP-PVA induced suppression (Table 29, A., Gps. 4 and 6 respectively), indicating that the route of administration of the NIP-PVA was not critical in this system.

b) In contrast, Cy treatment did not affect suppression by 1 mg of NIP-PVA (Table 30, A. and B., Gp. 1 v. 2). Most likely the higher dose of NIP-PVA tolerized B cells directly, an effect not reversible by Cy.

Milligram doses of NIP-PVA consistently could not suppress the anti-OA responses of mice immunized 1d later with NIP-OA (Table 10, B. and Table 11.). However, *in vivo* and *in vitro* F/T_E sometimes inhibited the anti-OA as well as the anti-NIP responses (Table 28 and

Table 30. Effect of cyclophosphamide on suppression by free NIP_4-PVA.

	Pre-treatment with Cy on d-4	Dose of NIP-PVA on d-1	Route of Administration	ANTI-NIP[3] PCA TITERS d10	d14
A. 1.	+	1 mg	i.p.	<20	<20
2.	-	1 mg	i.p.	30	<20
3.	+	100 ng	i.v.	205	410
4.	-	100 ng	i.v.	105	105
5.	-	10 μg	i.v.	220	70
6.	-	100 ng	i.p.	30	55
7.	-	-	-	825	780
8.	-	-	-	360	200
				545[1]	405[1]
B. 1.	+	1 mg	i.p.	<20	<20
2.	-	1 mg[2]	i.p.	30	25
3.	+	50 ng[2]	i.v.	360	105
4.	-	50 ng[2]	i.v.	55	50
5.	-	10 μg	i.v.	60	55
6.	-	50 ng	i.v.	105	60
7.	-	-	-	825	210
8.	-	-	-	410	200
				580[1]	205[1]

[1] Geometric mean of titers for controls receiving only NIP-OA+$Al(OH)_3$
[2] Including $2x10^7$ cell equiv. of F/T_F from normal spleen cells
[3] Recipients immunized with 3 μg NIP_4-OA+$Al(OH)_3$ on d0

Table 18). Possibly, in the case of mg doses of NIP-PVA, only direct B cell tolerance might be operational, limiting the effect to B cells binding NIP-PVA. In the case of ng doses of NIP-PVA, T suppressor cells might be induced, allowing under special conditions for antigen (NIP-OA) bridging to occur between NIP-specific T_s cells and OA-specific B cells or T_h cells [a type of cognate suppression, mentioned by Dorf and Benacerraf (32a)], with the final effect being antigen-nonspecific.

NIP-PVA at 50 ng was injected i.v. together with the F/T_E of normal spleen T cells. The effect of this procedure was identical to that of the transfer from mice treated with NIP-PVA of spleen T F/T_E containing about 50 ng of NIP-PVA. Furthermore, the amount of NIP-PVA carried over by the spleen cells 7 days later (less than 1% expected from the radiolabel studies in Table 21) was well below the minimum required for a suppressive effect.

Transferable suppression was not detectable in the spleens of the mice treated with 50 ng of NIP-PVA 7d earlier (data not shown). For suppressor cells to be generated with such a low dose of NIP-PVA on d0, it might have been necessary to immunize the donors on d1 or d2 with NIP-OA, in order to promote the expansion of NIP-specific suppressor cells. In the experiments with Cy treatment, suppressor cells may have been induced in this fashion, as the mice were immunized 1d after the treatment with NIP-PVA. On the other hand, 50 ng of NIP-PVA treatment alone may not have sufficed to generate enough suppressor cells for detection by cell transfer, although these might have been operative in the donors.

DISCUSSION

At the time when this investigation was initiated, hapten-specific suppressor cells had not been well-characterized. However, other workers in this laboratory (60,61) had succeeded in inducing hapten-specific suppression of IgE responses by the corresponding hapten-PVA conjugates. Hence, the hapten-PVA system appeared to be a potentially useful model for the further delineation of hapten-specific T_s cells, in terms of their activation requirements and effector functions. Already DNP-PVA had been shown to activate suppressor T cells (48) and suppressor factor(s) had been isolated from these cells (49,42). Moreover, since IgE responses constitute examples of overt immune responses which might be downregulated with PVA conjugates of allergenic determinants, it seemed desirable to elucidate the mechanism(s) of suppression in the hapten-PVA system.

Based on previous work in the laboratory of Dorf and co-workers (62), the haptens NP and NIP were chosen for this investigation. In the C57BL/6 strain of mice used in these studies, the antibodies to NP were predominantly of one idiotype, NP^b. Should suppressor T cells have been generated, one could have also examined the involvement of idiotypic interactions between suppressor T cell subsets. The C57BL/6 strain of mice that was chosen for this project was known for a predominant idiotype (NP^b) characterizing its primary response to NP. A further goal was to isolate the suppressor factor(s) which might mediate the suppression of IgE and IgG responses to NP and NIP.

Before embarking on the study of the NP/NIP system, a link with

previous work in this laboratory was established by reproducing certain findings of Lee and Sehon, namely the transfer of suppression from DNP-PVA treated donors and the isolation of suppressor factor(s) from donor spleen cells. Also, to imitate a situation that might be found in allergic patients presensitized to allergens, mice with an ongoing IgE response to haptenated-OA were treated with hapten-PVA conjugates at the time of the secondary exposure to hapten-OA. As illustrated in Tables 1.a) and b) (for the hapten DNP) and in Table 2 (for the hapten Flu), the suppression of the secondary IgE response to DNP-OA and Flu-OA was highly significant. A difference of 4-fold or more between PCA titers was considered significant, since the PCA titer of a given sample is accurate to within a factor of 2 (± one 2-fold dilution) (51).

The suppression of the secondary responses by hapten-PVA conjugates was most likely due to a direct tolerogenic effect of the hapten-PVA conjugates on the memory B cells. Since the secondary responses peaked at day 7 (Tables 1.a),b) and Table 2), it would be unlikely for suppressor cells to be induced and expanded sufficiently by 6-8 days following hapten-PVA treatment, for these cells to have a noticeable effect on the activity of the secondary memory B cells. It is well known in various suppressor cell systems (16, 27, 32a) that 4 to 7 days are required for the detection of suppressor cells of the first order (Ts_1).

A more suitable procedure for inducing suppressor T cells would involve the injection of hapten-PVA conjugates into naive mice, the method followed by Lee and Sehon (48) for the DNP hapten. Thus it was confirmed in numerous experiments (see Tables 3, 4, 5, 6) that

the spleen cells of mice treated with hapten-PVA, but without having been exposed to the immunogenic hapten-OA conjugate, were capable of inducing suppression with an interval of 5-22d after treatment.

Preliminary results in this laboratory by Mr. John Jeffrey with NP-PVA as the tolerogen, also had indicated that suppression of the tolerized mice could be transferred via NW T cells. However, in this system, it was obligatory to immunize the donor mice one day after treatment with tolerogen in order to induce transferable suppression (data not shown) in BDF_1 mice. The inclusion of the immunogen in the treatment might have helped to expand NP-specific suppressor cell clones induced by NP-PVA. In a preliminary experiment this author (S.K.) found that NIP-PVA alone also did not engender transferable suppression in BDF_1 mice, suggesting a similar requirement for the synergistic effect of the immunogen in the induction of suppressor cells specific for the NIP hapten.

Before proceeding with the proposed studies of suppressor cells and factor(s) in the NP/NIP system, the effect of NIP-PVA conjugates on the primary IgE response to NIP-OA was tested (Table 10). Thus, treatment of naive BDF_1 mice with NP- or NIP-PVA three days and one day before primary immunization was sufficient to suppress the NP- or NIP-specific responses in these mice. NIP was chosen for further work since it could be expected that in the C57BL/6 strain the predominant idiotype against NP, also known as the NP^b id, would bind with higher affinity to NIP. The ability of NIP-PVA to abrogate primary responses to NIP-OA was examined in two other strains of mice, C57BL/6 and BALB/c (Table 11). A state of nonresponsiveness was induced in both these strains.

Previous results obtained with the NP/NIP system in BDF_1 mice also suggested treating the donors of putative suppressor T cells with immunogen as well as tolerogen. One more experiment in BDF_1 mice (Fig. 5) confirmed the previous findings in this laboratory that the combination of immmunogen and tolerogen was successful in inducing transferable suppression. It was assumed that the same treatment would apply to C57BL/6 mice.

There was a precedent in the literature (62) for the binding of NP-specific suppressor T cells to NP-BSA-coated plastic dishes, and, therefore, following the same method, attempts were made to isolate NP- and NIP-specific cells by binding spleen T cells from NP- or NIP-PVA treated C57BL/6 mice to the plates and removing them with cold shock. However, no functional suppressor cells were detectable, even though the yield of hapten-binding cells was 5-10% of the cells plated. One explanation for this result was clearly that there may have been no suppressor cells induced by NP- or NIP-PVA. Also it should be noted that in all these experiments to test for the enrichment of suppressive T cells on NP- or NIP-BSA coated dishes, the donor mice had been injected with both tolerogen and immunogen 7 days before transfer. As was shown in Tables 12 and 13, the inclusion of an immunogenic stimulus invariably prevented the transfer of suppression in C57BL/6 mice. Therefore, to favour the transfer of suppression, the immunogen was omitted from the treatment of donor mice in all future experiments. Clearly, in the C57BL/6 strain NIP-PVA alone was sufficient to give transferable suppression.

To pursue further the hypothesis that suppressor cells were induced by treatment with NIP-PVA, the F/T extracts of spleen T cells

from NIP-PVA treated C57BL/6 were subjected to analysis. It had already been shown in the DNP and FLu systems that the F/T extracts were as efficient as the cells from which they were derived in transferring suppression of a primary response to the appropriate hapten in recipient mice (Tables 3,4 and 5). As suppressor factor(s) had been reported by other laboratories (see LITERATURE REVIEW), it was reasonable to pursue the characterization of the suppressive agent in the above F/T_E. In the DNP system, several approaches were used to determine the properties of the suppressive entity. Precipitation at 50% of SAS resulted in the recovery of suppressive activity in the precipitate, as would have been expected from a protein (Table 7). Fractionation of a similar extract on a G-75 column (which chromatographs proteins less than 80 kD) resulted in the recovery of suppressive activity in the void volume, representing a molecular weight of greater than 60 kD (Table 9 and Fig. 4). The molecular weights reported for suppressor factor(s) were in the range of 40-70 kD, so this result did not rule out a suppressor factor as the agent of suppression. It was noted that a non-specific suppression was induced by all the extracts, even from the control spleen cells, emphasizing the crude nature of such cell extracts containing several possible materials with biological effects. However, the suppression by the experimental group 1 was still at least 4-fold relative to the control group 2. The effects of non-specific suppression must be taken into account whenever cells or cell extracts are injected, thereby necessitating a control group of cells from untreated mice or from mice treated with only the solvent normally employed for hapten-PVA conjugates. Although the cells injected into recipient

mice were always syngeneic, the mere introduction of cells or their extracts i.v. may have perturbed the fine balance of the recipient's immune system. However, this should still allow for hapten-specific effects to be detected over and above the antigen non-specific effects.

With the C57BL/6 strain, the F/T_E also retained the suppressive activity of the appropriate NW spleen T cells (Table 12 and 13), so it seemed reasonable to attempt the isolation of the putative suppressor factor(s) on immunoadsorbent columns (117). The Seph 4B gels were coupled to either NIP-BSA for removing NIP-specific factors or B6anti-NP, which would bind anti-idiotype suppressor factors. The first two experiments (data not shown) indicated that the suppressive activity passed through both NIP-BSA-4B and B6anti-NP-4B, which was puzzling due to the hapten-specificity of the suppression (Table 15). The method of incubating samples with the Seph 4B was later improved by eliminating NMS from the loading and washing buffers, as well as using a batch method of incubation as opposed to columns. As a result of this change, the suppressive activity was absorbed by the B6anti-NP, but still did not bind to NIP-BSA.

Since NIP-BSA-4B could not deplete the F/T_E of suppressive activity, a strong possibility was that the suppressive activity of the F/T_E was actually NIP-PVA carried over from the original donors, although it was also remotely possible that an anti-idiotypic suppressor factor had been extracted from the spleen T cells of the NIP-PVA treated mice. To pursue the carry-over hypothesis, it was necessary to label the NIP-PVA for tracer studies, capitalizing on the iodine naturally present on the NIP hapten, i.e. to synthesize NIP with

^{125}I. Such tagging had not been possible for the other haptens DNP, BPO or FLu which were studied previously, therefore providing a unique opportunity to investigate the contribution of carry-over to the transfer of suppression engendered by conjugates of NIP-PVA.

Several approaches were taken to rule out the presence of suppressor cells and to test for the hypothesis of carry-over. The question of carry-over has been addressed by others (54,66,67) as a possible mode of transfer of suppression, but was never implicated, either because of kinetics of the suppression or because the antigen carried over was ineffective at the dose transferred by a suppressor factor preparation. A propensity of PVA for membrane surfaces could be expected from its partially hydrophilic nature. Indeed, Tables 16 and 17 showed that the carry-over of NIP-PVA into the spleen was such that the level of NIP-PVA in the spleen remained fairly constant over the time of study. The persistence of NIP-PVA in these mice would keep the spleen T cells exposed to NIP-PVA, resulting in carry-over when the spleen T cells were transferred. Later experiments with the F/T_E of these spleen T cells (Table 18) showed that the NIP-PVA by itself, in the same amount as found in the F/T_E, was sufficient to induce tolerance in recipients, even without the inclusion of the cell extracts. The results reported here emphasize that one must rule out the carry-over of suppressive agents before attributing suppression to a suppressor cell or suppressor factor(s). Carry-over was reported in another system by Middleton *et al.* (74), wherein spleen cells tolerized *in vivo* by DNP-polyacrylamide could inhibit the immune response of naive spleen cells *in vitro* by virtue of the release of bound DNP-polyacrylamide from the donor cells into the

culture medium.

Furthermore, the existence of the suppressor cell itself has been questioned, most recently by Möller (103). He concludes that since less is known about suppressor T cells and their receptors for antigen, than is the case for T cells of both the helper and cytotoxic phenotypes, he prefers to dismiss the existence of T_s cells. Moreover, the existence of the I-J gene has been cast into doubt, thereby leaving suppressor cells without a "genetic address." However, in the NIP-PVA system, although suppressor cells were not detected in mice treated with NIP-PVA, this does not refute their very existence, but merely provides an alternative explanation to the phenomenon of transfer of suppression. Under the appropriate conditions, clearly, some form of suppressive cell specific for NIP could be induced (32a); whether or not one wishes to call it a suppressor cell, or another form of cytotoxic cell as some immunologists would argue, is a moot point at this time.

The effect of the immmunogen on the transfer of suppression in Tables 12 and 13 could now be explained in a new light. NIP-specific antibodies produced in the donor by the NIP-OA might have increased the clearance rate of NIP-PVA from circulation, thereby leading to a reduction in the amount of NIP-PVA carried over on spleen T cells. Another factor could have been the masking of NIP-PVA by the same NIP-specific antibodies. Despite the NIP-PVA treatment, some low affinity anti-NIP antibody producing cells might have escaped tolerance.

The explanation of NIP-PVA carry-over was further supported by the results of the experiments in Tables 19 through to 23. In these

experiments the following questions were answered: 1) how early after treatment with NIP-PVA could the suppression be transferred from the donor mice? (Carry-over would be favoured by an early transfer;) 2) Could anti-Thy1.2 plus complement or irradiation abolish the transfer of suppression? (Only a suppressor T cell, not NIP-PVA carry-over, would be sensitive to these treatments;) and 3) Could the suppressive activity be absorbed onto a column of B6anti-NP-4B? (B6anti-NP should be able to bind the NIP on the PVA.) In Table 22, to answer question 1), it was shown that only 2 days after the treatment with NIP-PVA, suppression could be transferred, favouring carry-over as a mechanism of transfer. Two days is not normally enough time for suppressor cell induction (16, 27, 32a) although for one experiment reported by Lee *et al.* (66) with NIP-poly(N-vinyl-pyrrolidone) (PVP) as the suppressive agent, it was mentioned in the discussion that suppression could not be transferred before two days following NIP-PVP treatment, ruling out carry-over as a mechanism of transfer and arguing in favour of suppressor cells as early as two days after NIP-PVP injection. Question 2) was answered in Table 23.a) and b), to the effect that neither anti-Thy1.2 plus complement nor irradiation was able to abolish transfer of suppression, again supporting the hypothesis of carry-over. Finally, to answer question 3), it was clear from Tables 19 and 20 that not only free $N^{125}IP$-PVA, but also suppressive activity in a F/T_E could be bound by an anti-NP immunoadsorbent, again suggesting that it was the NIP-PVA in the extract could be responsible for the suppressive effects of the F/T_E. In contrast, NIP-BSA-Seph 4B did not remove the suppressive activity in the F/T_E (Table 21), which was to be expected if the suppressive

entity was NIP-PVA carried over in the F/T_E.

To answer more questions about the carry-over of NIP-PVA, a reproducible method was established to pulse NIP-PVA onto spleen T cells *in vitro* (Fig. 6, 7 and 8), without allowing for suppressor cell induction due to the short time of incubation. It became possible to compare the amount of NIP-PVA found in association with spleen T cells with the same amount of free NIP-PVA, in terms of suppressive effects on the NIP-specific IgE response. Since free NIP-PVA was able to suppress primary NIP-specific IgE responses at the same low doses carried over in the F/T_E (Table 18), it was hypothesized that however NIP-PVA was carried over (in the cytoplasm or on the membranes of spleen T cells) it finally was released in the free form upon freezing and thawing of the cells. Since no cell-related components were required for the suppressive effect of nanogram doses of NIP-PVA, it was not surprising to discover a lack of genetic restriction in the activity of a F/T_E from C57BL/6 mice. Thus, even when the recipient strain of mouse was incompatible at the H-2 or Igh-V regions, suppressive activity could be transferred. Dorf and co-workers (32a), in contrast, found that in the NP system all their suppressor factors, i.e. TsF_1, TsF_2 and TsF_3 were either I-J- or Igh-restricted in their activities, requiring compatibility between the donor and the recipient mice. It should be remembered that the induction of suppression in their system was by NP-modified syngeneic adherent cells expressing I-A or I-J histocompatibility antigens, while in this system with NIP-PVA, no cell association was required for the NIP-PVA to induce suppression. If the tolerogenic F/T_E, however, was artificially pulsed onto splenic adherent cells,

as was shown in Table 25, suppression could also be transferred with the adherent cells. It is possible that the NIP-PVA from the F/T_E became attached to the adherent cells, thus making them capable of inducing suppressor cells in the recipients as in the NP system (32a). Furthermore, the very nature of PVA, an amphipathic molecule potentially capable of intercalating with cell membranes, suggested a role of cell-bound PVA-hapten conjugates in the transfer of suppression.

In the instances where the anti-OA response was also affected by F/T_E (Tables 22,18 and 28) one could envisage that some form of NIP-specific suppressor cell was induced in the recipient of low doses of NIP-PVA. Subsequently, the antigen used to immunize the mice, either NIP_4-OA or NIP_8-OA, could link an OA-specific T helper cell or B cell to the NIP-specific suppressor cell, resulting in a form of cognate and non-specific suppression of the target cell. Such non-specific suppression has been reported by Kishimoto *et al.* (75), who reported that the suppressor T cell primed by DNP-Mycobacterium had to be induced by DNP, but that it could suppress non-specifically a response to an unrelated hapten, BPO, in an IgE-restricted fashion. Therefore, the factor(s) released by this suppressor T cell contained a class-specific as opposed to a hapten-specific suppressor factor.

Another aspect of the carried-over NIP-PVA in the F/T_E was its physical properties as determined by filtration on both XM-100A and XM-300 ultrafiltration membranes (Table 27) compared to free NIP-PVA (Table 26) in the presence of F/T from normal spleen cells. Both forms of NIP-PVA were identical in that they passed though the XM-300

but were retained on the XM-100A, indicating identical molecular weight characteristics. This finding was complemented by Table 28, which showed that the suppressive activity in the F/T_E was also retained on the XM-100A filter.

Another approach to comparing free NIP-PVA with the F/T_E was chromatography on a column of ACA-44 (Table 29). It was found that the suppressive activity appeared in a fraction which overlapped with the profile for free NIP-PVA (Fig. 9 vs. Fig. 10), thereby suggesting that NIP-PVA could certainly be responsible for the suppressive activity in the F/T_E.

In the TM-PVA system (99) no evidence was found for the activation of T_s cells, which suggested that different haptens coupled to PVA may differ in their ability to induce hapten-specific T_s cells. This observation supports the relevance of testing another hapten, NIP, coupled to PVA for the possible induction of T_s cells. 7d after NIP-PVA treatment of donor mice, the amount of NIP-PVA carried over on $2x10^7$ T cell equivalents (in the range 10-100 ng) was sufficient to induce suppression by itself, and this explains the transfer of suppression without invoking suppressor T cells. When TM-PVA was used as a tolerogen (47), however, no form of suppression could be transferred, ruling out significant carry-over in that system. Instead B cells were directly tolerized by TM-PVA.

The differences between the various hapten-PVA conjugates as to their mechanism(s) of suppression could be attributed to the level of previous exposure of the mice to environmental antigens cross-reacting with the different haptens. DNP is cross-reactive with several environmental determinants; therefore, several suppres-

sor T cell clones could have been induced by DNP-PVA. In the light of the evidence with different hapten-PVA conjugates, it appears that the criteria for suppressor T cell induction may be different for each hapten. Although mg amounts of DNP-PVA appeared to induce suppressor T cells specific for DNP (61), it remains to be answered how this induction was brought about. Possibly, DNP-PVA, either in the free form or presented by another cell such as a macrophage, induced a DNP-specific precursor T_s cell. Suppressor factor(s) produced by these suppressor cells could induce a higher order of suppressor cell leading towards an effector suppressor cell. This effector cell could bind to a DNP-specific target cell through a hapten-hapten bridge (since the immunogen DNP_3-OA had more than one hapten). One reason that hapten-PVA conjugates are non-immunogenic may be that they cannot be processed by antigen-presenting cells, since PVA is an artificial polysaccharide, not easily catabolized (also explaining its persistence in various anatomic compartments after i.p. injection). To further underline the difference between activation of suppressor cells and helper cells (tolerance vs. immunity), the splenic adherent cells responsible for inducing Ts_1 in the NP system are UV-B resistant (83), in contrast to antigen-presenting Langerhans cells and splenic dendritic cells, both of which become defective in antigen presentation upon UV-B irradiation (101, 102). It is not known what stage of antigen presentation is affected by UV-B and is required for the stimulation of T_h or T_{DH}. However, the conditions for stimulating T_s appear to be less stringent.

All hapten-PVA conjugates studied thus far (42,46,99) consistently could tolerize B cells directly. To distinguish between B cell

tolerance and suppressor T cell induction in the recipients of nanogram doses of NIP-PVA (i.e. carried-over NIP-PVA), these recipients were treated with Cy 3 days before injection of the tolerogen (Table 30). Suppressor cell precursors would be expected to be especially sensitive to Cy (76). The results, although preliminary, indicated that nanogram doses of NIP-PVA induced a form of suppression which was reversible with Cy, while the suppression by 1 mg of NIP-PVA was resistant to Cy treatment. Direct B cell tolerance might be the only mechanism for suppression by 1-2 mg of NIP-PVA, although suppressor cells might have co-existed with the tolerized B cells, and simply escaped detection by the various procedures used in the above experiments. It appeared that the suppression induced by 50-100 ng of NIP-PVA was not due to B cell tolerance, but rather due to the induction of suppressor cells in the recipients. However, the reversal of suppression by Cy in Group 3 (Expts. A and B) might have been due to removal of non-specific suppressor cells. The control that was missing to properly examine this possibility was the effect of Cy on the control responses (Groups 7 and 8). It is possible that Cy enhances the control response, and also reverses a weak tolerogenic effect with low doses of NIP-PVA but that this effect would not be evident in the face of overwhelming B cell tolerance induced with 1 mg of NIP-PVA.

The effect of low and high doses of tolerogen was also studied by Eichmann and co-workers. In their system, the A5A idiotype positive portion of the response of A/J mice to Group A streptococcal carbohydrate (A-CHO) was manipulated with guinea pig IgG anti-A5A (anti-idiotype) (71). They showed that low-zone (100 ng) suppression

with the anti-id required 5-7 wks for the induction of transferable suppressor cells. The target of these suppressor cells was the A5A+ T helper component of the anti-A-CHO response. In the case of high zone (60 μg) treatment, suppression could not be transferred. On the contrary, both B cell precursors and T helper cells were directly suppressed. Malley (88) also could induce antigen-specific suppressor T cells with 1-10 μg of a rabbit anti-idiotypic antibody.

In summary, the previous method of inducing suppression in mice with mg amounts of NIP-PVA does not lead to demonstrable suppressor cells in C57BL/6 mice, while lowering the dose to the ng level might induce suppressor cells. It might be worth testing some of the ideas presented here by a future study involving all three haptens, DNP, TMA and NIP simultaneously in the same strain of mice in order to properly assess the differences between them. Also one may envisage that NIP-PVA could still induce T_s under other conditions such as the injection of immunogen at an appropriate time after tolerogen. The time between tolerization and transfer of the spleen T cells might also have to be lengthened to allow for suppressor cells to be expanded in the donor mice. Finally, in terms of the tolerogenic effect of NIP-PVA, the model of NP-secreting hybridomas could be used to study the mechanism of tolerance at the level of the immortalized plasma cell.

CONTRIBUTIONS TO KNOWLEDGE

1) The results reported herein include the first documented report of suppression achieved by the treatment of C57BL/6 and BALB/c mice (low IgE responders to haptenated-OA) with conjugates of PVA and the hapten, NIP. Previous reports involved treatment of BDF_1 mice (a hybrid strain with high IgE responsiveness to hapten-OA) with conjugates of PVA and either DNP, BPO or TM.

2) It was demonstrated in this study that transfer of suppression by spleen T cells could be attributed to the carry-over of the original suppressive agent (in this case PVA-hapten conjugates) rather than to the induction of suppressor T cells. To reach this conclusion a new method was devised to radiolabel NIP-PVA for tracer studies. Furthermore, a thorough analysis was carried out of the anatomic distribution of NIP-PVA over a one week period after i.p. injection.

3) This report is the first to suggest a dose-dependency of the type of suppression by hapten-PVA conjugates. There was evidence for direct tolerance of hapten-specific B cells in the C57BL/6 strain of mice treated with 1-2 mg of NIP-PVA, while the suppression by lower doses of 50-100 ng of NIP-PVA was apparently Cy sensitive, which was interpreted as being due to T cells.

4) The discovery of the effectiveness of nanogram amounts of NIP-PVA could be advantageous in the potential application of this

suppressive agent (PVA coupled to allergenic determinants) to human allergies. A potent tolerogen effective at low doses would have few side-effects. If suppressor T cells indeed were favoured by the lower dose of NIP-PVA injected into mice, one might expect the same for humans, resulting in a longer and more efficient means of suppression. Direct B cell tolerance, aside from requiring higher doses, would also be reversible by recruitment of new progenitor B cells from the bone marrow, following clearance of the tolerogen from circulation.

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