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We report here a description of the synthesis of a new hapten-polymer conjugate, 3-(2,4-dinitrobenzene sulfonyl) polyvinyl pyrrolidone (DNP-PVP). The sulfonyl-DNP ligand was appended to the pyrrolidone unit via the reaction of the lithium enolate of PVP with the chloride analog of dinitrobenzene sulfonic acid. The relationship between epitope density and immunogenicity of this conjugate was explored and we have found that a minimum of 24 moles of DNP per PVP is necessary in order for a significant anti-DNP response to occur. This conjugate is able to induce a significant anti-DNP antibody response both *in vivo* and *in vitro*. Experiments done with congenitally athymic mice indicate that the DNP-PVP conjugate is a T-independent antigen. Studies previously reported in the literature have shown that preimmunization of mice with a non-immunogenic, low molecular weight preparation of PVP (10 kd; PVP_{10}) suppresses the response to later injections of an immunogenic, higher molecular weight form of PVP (360 kd; PVP_{360}). Experiments reported here indicate that animals primed with PVP_{10} exhibit a reduction in both

the anti-hapten and anti-carrier antibody response to $DNP-PVP_{360}$.
We suggest that $DNP-PVP_{360}$ may prove to be a valuable tool for
further elucidation of the nature and specificity of the PVP-
suppressor system.

In addition, studies were conducted to investigate the possible
role of prostaglandin synthesis in regulating the PVP_{10}-induced
suppressor cell population. Results indicate that PVP_{10}-induced
suppression is not affected by prostaglandin synthetase inhibitors.
However, it also appears that prostaglandin synthesis inhibition
alone is capable of mimicking the activity of the induced suppressor
cell population. The preparation of the $DNP-PVP_{360}$ conjugate should
permit further elucidation of the nature of PVP-induced suppression
to be carried out in vitro with isolated cell populations.

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Redacted for privacy

Chairman of the Department of Microbiology

Redacted for privacy

Dean of Graduate School

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Typed by C.R. Adams-Burton for   Catherine Ruth Adams-Burton

For Bud, Margaret and Ruth,

whose teachings, example and faith

nurtured my inquisitive nature.

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I. REVIEW OF PERTINENT LITERATURE

Lymphocyte populations

One of the principal advances in immunobiology has been the rec-
ognition of the differentiation of lymphocytes into two separate popu-
lations. All lymphocytes arise from the bone marrow, but mature and
differentiate through separate pathways. The B lymphocyte population
migrates to distinct sites in peripheral lymphoid tissues where they
are transformed into the precursors of antibody-producing cells. The
second lymphocyte type, the thymic or T cell population, pass through
the cortex of the thymus, proliferate, and differentiate into mature
lymphocytes capable of mediating the cellular types of immunity. The
cellular reactions include delayed type hypersensitivity, transplan-
tation reactivity and acquired cellular resistance to infection. In
addition, a subpopulation of this cell type is responsible for the
regulation of various functions of other cells in the immune system
(Claman & Chaperon, 1969; Davies, 1969; Miller & Mitchell, 1969; Katz
& Benacerraf, 1972). These regulatory T cells may be further divided
into a subpopulation with enhancing (or helper) activity and a subpop-
ulation with inhibiting (or suppressor) activity. Thymic helper cells
are defined as T cells which augment the ability of B cells to produce
antibody. Thymic suppressor cells are those which inhibit the antibody
producing capacity of B cells, as well as activities of other T cells.

Cantor and Boyse (1977) have described a series of membrane differentiation antigens for mouse lymphocytes which are termed Ly antigens. Three of these antigens, Ly 1, 2 and 3, are expressed exclusively on T lymphocytes and thus are termed Lyt antigens. Three distinct subclasses of T cells have been identified based on differences in the expression of Lyt antigens (Cantor & Boyse, 1977). Lyt $1^+2^+3^+$ cells comprise approximately 50 percent of the peripheral T cell population. These cells are the first to appear during ontogeny and at least a portion of the Lyt $1^+2^+3^+$ cells are depleted by adult thymectomy and by low doses of cyclophosphamide (Cantor & Boyse, 1977; Eardley et al., 1978). The Lyt $1^+2^+3^+$ cells apparently function as amplifier cells in the immune response and have been shown to augment both suppressor and helper T cell activities (Feldmann et al., 1977; Tada et al., 1977; Eardley et al., 1978; Muirhead & Cudkowicz, 1978). A second subclass of T cell comprises approximately 30 percent of the peripheral T cell population and has the phenotype Lyt $1^+2^-3^-$. These cells mediate delayed type hypersensitivity responses and function as helper cells for antibody production and activation of suppressor cells and cytotoxic cells (Cantor et al., 1976; Cantor & Boyse, 1977; Eardley et al., 1978). The third subclass of T cells comprise approximately five to ten percent of the peripheral T cell population, display the Lyt $1^-2^+3^+$ phenotype and function as cytotoxic killer cells and suppressor cells (Cantor et al., 1976; Cantor & Boyse, 1977).

Antigens associated with the I-region of the mouse major histocompatibility complex (Ia antigens) are found on the surface of most

mouse B cells and also on some T cells and macrophages. Further

delineation of functional subsets of T cells has been possible based

on the presence or absence of these antigens. For example, at least

two classes of Lyt 1$^+$ T cells are required to provide optimal helper

activity to B cells for antibody production after T-dependent antigen

stimulation (Cantor et al., 1978; Tada et al., 1978). One of these

Lyt 1$^+$ cells is nonadherent to nylon wool and Ia$^-$ while the other

is adherent to nylon wool and Ia$^+$ (Tada et al., 1978). Similarly,

within the Lyt 2$^+$3$^+$ T cell population, T suppressor cells have been

shown to be Ia$^+$(I-J) while cytotoxic T cells appear to be Ia$^-$ (Beverley

et al., 1976; Okumura et al., 1976).

Suppressor thymic lymphocytes

Two groups of investigators, working independently, have been

credited with first demonstrating T lymphocyte suppressor activity.

Baker and associates demonstrated the enhancing effect of anti-lymphocyte

serum (ALS) on the antibody response to the T-independent antigen,

pneumococcal polysaccharide (SSS-III). Briefly, they found that

this enhancement was abrogated by passive transfer of normal thymocytes,

that low-dose tolerance to SSS-III was blocked by ALS treatment and

that the response of athymic mice to SSS-III was not affected after

treatment with ALS (Baker et al., 1970, 1973, 1974a, 1974b; Barthold

et al., 1974). Gershon and Kondo (1970, 1971) established that when

mice were injected with high dosages of sheep erythrocytes, an immuno-

tolerant state was induced. This tolerance could not be induced in the

absence of T cells. These findings have led to further studies on

the role of suppressor T cells in the immune response.

The mechanism of T suppressor cell function is not fully understood and several models explaining the function of these cells have been proposed. These models are based on mechanisms which include direct suppressor cell to target cell contact, release of soluble suppressor factors, or a combination of both. Several soluble suppressor factors have been isolated and characterized to date. These include both antigen-specific and nonspecific factors. One of the first antigen-specific soluble suppressor factors isolated was described by Tada and associates (1974). They were able to isolate a factor from sonicated thymocytes or from spleen cells of keyhole limpet hemocyanin (KLH)-primed rats that was capable of suppressing IgE responses to DNP-KLH. The factor, possessing a molecular weight between 35,000 and 65,000 daltons, may be adsorbed by the carrier used to generate it. This factor shares antigenic determinants with thymocyte membrane antigens but does not cross-react with immunoglobulin antigenic determinants. Genes in the I-J region of the H-2 locus appear to code for the suppressor factor.

An antigen-specific suppressor factor which inhibits the antibody response of B cells to the synthetic terpolymer of L-glutamic acid, L-alanine and L-tyrosine (GAT) has been isolated from a nonresponder strain of mice (Kapp et al., 1976). The factor is a 45,000 dalton glycoprotein, shares antigenic determinants with the Ia antigens, binds to GAT and does not contain immunoglobulin markers.

A concanavalin A (con A)-induced suppressor factor has been described (Rich & Pierce, 1973, 1974; Rich & Rich, 1975; Pierce &

Kapp, 1976) which nonspecifically suppresses in vitro plaque forming responses to T-dependent and T-independent antigens and is termed the soluble immune response suppressor (SIRS). The SIRS is able to suppress in vitro mitogenic stimulation of B cells by lipopolysaccharide (LPS) but not mixed lymphocyte reactions or generation of cytotoxic lymphocytes (Peavy & Pierce, 1974; Rich & Rich, 1975; Pierce et al., 1976). The SIRS appears to be approximately 35,000 to 67,000 daltons in size and produced by a Lyt 2^+3^+ lymphocyte (Pierce & Kapp, 1976).

Rogers and associates (Webb et al., 1979, 1980b; Rogers et al., 1980) have isolated soluble suppressor factors by culturing glass adherent splenic T cells in the presence of prostaglandin E_2. This factor, designated prostaglandin-induced-T-cell-derived suppressor (PITS), nonspecifically suppresses phytohemagglutinin (PHA) and LPS induced mitogenesis. The PITS is resistant to boiling and treatment with RNase and DNase, yet is sensitive to treatment with trypsin, proteinase K and Pronase. Analysis of PITS-containing supernatants shows that two distinct factors are present: $PITS_\alpha$, a 35,000 dalton factor and $PITS_\beta$, a 2000 to 5000 dalton factor. It appears that prostaglandin E_2 induces the T suppressor cell to release PITS rather than activate the cell to synthesize PITS.

Several other suppressor factors have been isolated and analyzed. Most of these share the functional and structural characteristics of those reviewed here. It seems apparent that further analysis of these molecules will help to clarify the mechanisms by which T suppressor cells function.

Prostaglandins

Several investigators have suggested that prostaglandins, prin-
cipally the PGE and $PGF_{2\alpha}$ series, play a role in the regulation of
the immune response. Investigations of the possible role of cyclic
AMP (cAMP) and PGE as second messengers in the activation of lympho-
cytes have led to the discovery that PGE_2 is capable of suppressing
phytohemagglutinin (PHA)-stimulated DNA synthesis in human peripheral
blood lymphocytes (Bourne et al., 1974; Parker et al., 1974). Further
studies have shown that prostaglandins added exogenously to lympho-
cytes in vitro reduce the expression of a number of immune functions
(reviewed in Webb et al., 1979). On the other hand, secondary (anam-
nestic) antibody responses are enhanced by PGE (Cook et al., 1978).
In general, prostaglandin synthetase inhibitors tend to induce an
improvement in the amplitude of the immune response (reviewed in
Webb et al., 1979).

The mechanism by which prostaglandins regulate these responses
has been investigated at both the intracellular level and the molecular
level. At the molecular level, it has been shown that mitogen- or
antigen-stimulated lymphocytes and macrophages release prostaglandins
(Webb & Osheroff, 1976; Grimm et al., 1978; Webb & Nowowiejski, 1978).
This antigen-induced elevation in prostaglandin levels in turn elevates
the intracellular cAMP level (Osheroff et al., 1975; Plesia et al., 1975;
Webb et al., 1980a; Zimecki et al., 1980). Presumably, some or all
of the cAMP-sensitive, intracellular enzymes would be affected by
this elevation.

At the cellular level, it has been proposed that the synthesis of prostaglandins by lymphocyte subpopulations, stimulated by mitogen or antigen treatment, regulates the immune response of other lymphocyte subclasses (Goodwin et al., 1977; Webb, 1977). Evidence has also been presented that macrophages and lymphocytes are capable of auto-regulation by producing prostaglandins after antigenic stimulation (Gordon et al., 1976; Zimecki & Webb, 1976). Very recently, it has been demonstrated that prostaglandins are able to directly block lymphocyte transformation (Webb et al., 1979). In addition, PGE_2 is capable of inducing a class of glass-adherent T cells to produce a nonspecific suppressive peptide (Webb et al., 1979, 1980b; Rogers et al., 1980). These data have led to the theory that a feedback loop exists in which prostaglandin production by an activated T cell serves as the signal to induce a second T cell to release the suppressor peptide (Webb et al., 1979). This peptide would then regulate the ability of the activated T cell to pass through the cell cycle. Thus, it appears that prostaglandins serve as an early, fine tuning control in the regulation of T cell activation, and in certain kinds of B cell activation.

Thymus-independent antigens

Antigens which are capable of inducing antibody production in the absence of T "helper" cells are termed T-independent antigens. These antigens elicit a predominantly IgM antibody response in mice (Basten & Howard, 1973). However, both DNP-levan and DNP-ficoll induce variable numbers of indirect (IgG) plaque forming cells (Mosier

et al., 1974; Klaus & Humphrey, 1974). The response to primary immun-
ization with these antigens is rapid, with the peak splenic plaque
forming cell (PFC) response at day four or five, and in most cases,
a decline to low levels by day eight (Klaus & Humphrey, 1974).

Dinitrophenyl-levan, DNP-lipopolysaccharide (DNP-LPS) and DNP-
ficoll are among the most potent T-independent immunogens. These
materials elicit about 10^5 PFC per spleen at the peak of the response
(Klaus & Humphrey, 1975). Other T-independent antigens, including
pneumococcal polysaccharide (SSS-III) and polyvinyl pyrrolidone (PVP),
are much weaker immunogens (7,000 to 10,000 PFC/spleen; Baker et al.,
1970; Rotter & Trainin, 1974).

The immunoregulation of the response to T-independent antigens
is less complicated than the response to T-dependent antigens and,
for this reason, these antigens have received a great deal of attention.
Many investigators have suggested that an understanding of the critical
aspects of the structure and biological functions of T-independent
antigens will clarify the process of B cell activation. A variety
of mechanisms have been proposed to explain the interaction of T-
independent antigens with B cells. Three of them have received consid-
erable attention and are worthy of discussion.

Coutinho and Möller (1974) envisage that all B lymphocytes carry
"mitogen receptors" which represent the triggering sites on the cell
membrane. These sites would be triggered by some region of the antigen
molecule other than the antigenic determinant. In this model, the
principal role of the immunoglobulin receptor on the specific B cell

would be to form a high energy bond with the antigen, thus creating
a high local concentration of the antigen-triggering substance and
leading to the selective activation of specific B lymphocytes. As
the antigen is intrinsically stimulatory in this view, a sufficiently
high concentration would cause activation of the lymphocyte without
the requirement for specific immunoglobulin receptor binding. Thus,
T-independent antigens would be viewed as general B cell stimulants
or "polyclonal" activators, as is certainly true with LPS, and to a
lesser degree, SSS-III, the antigens employed in studies reported by
Coutinho and associates (Coutinho & Möller, 1973a, 1973b; Coutinho
et al., 1974). However, many T-independent antigens have proven
not to be polyclonal activators. Dinitrophenyl-levan and DNP-ficoll
have only very weak mitogenic ability while PVP and TNP-polyacrylamide
beads (TNP-PAA) have none (Andersson, 1969; Feldmann et al., 1974;
Klaus et al., 1975; Galanaud et al., 1976).

A second proposal on the nature of B cell activation states that
two independent signals are required (Bretscher & Cohn, 1970). One
such signal would be delivered through the interaction of the antigen
with the immunoglobulin receptor of the B lymphocyte and the second
signal might be delivered either by another portion of the same molecule
or by an independent moiety. Dukor and Hartmann (1973) proposed that
binding of activated C3 to complement receptors on B cells provided
the necessary second signal. It was envisaged that T-independent
antigens would generate this signal via their capacity to cleave C3
by the alternate pathway of complement activation. Although LPS,

levan and DNP-levan are potent C3 activators, DNP-ficoll, SSS-III

and DNP-SSS-III have no effect on C3 even at high concentrations

(Paul et al., 1974; Pryjma et al., 1974).

The third major proposal for B cell activation is that the pre-

sentation of antigenic determinants in an appropriate array or matrix

is the critical feature. In this view, the carrier moiety need not

deliver an independent signal or a second signal, but instead acts as

a passive carrier of antigenic determinants. Presentation of these

determinants in a critical spatial orientation would lead to cross-

linking of cellular surface receptors, which would in turn lead to

B cell activation (Feldmann & Basten, 1972). This hypothesis is

difficult to test since most T-independent antigens are polymeric

in structure and thus meet the requirements of the proposal. However,

data have been presented which argue against this concept (Langman

et al., 1974). These investigators found that the polymeric and

monomeric forms of the flagellar protein from a Salmonella strain

was able to induce an antibody response in T cell deprived mice while

the polymeric and monomeric flagellar proteins from a second Salmonella

strain was not. This suggests an important role of the individual

antigenic determinants rather than the three-dimensional structure

of the carrier in determining whether or not T cell participation

is required for B cell activation.

Although it was once thought that T-independent responses in-

volve only B cells, it has been well established that some subclasses

of T cells and possibly macrophages are also involved. Baker and

associates (Baker et al., 1970, 1973, 1974a, 1974b; Barthold et al.,

1974) have shown that a population of T cells exerts a suppressive

effect on the antibody response to SSS-III, although these findings

are somewhat controversial (Warr et al., 1975). In addition, several

investigators have demonstrated that a similar mechanism operates

in the response to PVP (Kerbel & Eidinger, 1971, 1972; Rotter & Trainin,

1974, 1975; Lake & Reed, 1976, 1977; Inaba et al., 1978a, 1978b).

Studies conducted with DNP-ficoll suggest the presence of a controlling

T cell population operating on the B cell response to this antigen

as well (Mosier et al., 1974; Paul et al., 1974).

The necessity for macrophages in the development of an antibody

response to several T-independent antigens remains an unresolved

issue (Mosier et al., 1974; Lee et al., 1976; Wong & Herscowitz, 1979;

Duclos et al., 1979; Kirkland et al., 1980; Boswell et al., 1980).

It would appear that the conclusions which have been drawn concerning

macrophage requirement can be correlated with the method used to deplete

macrophages from lymphoid cell populations. Although it is impossible

to unequivocally state whether macrophages are involved in T-independent

responses, it has become apparent that cell types other than B cells

play a functional and regulatory role and that the so-called T-inde-

pendent antigens are not truly independent of all influences from

thymic cell populations.

Polyvinyl pyrrolidone

The immunogenicity of polyvinyl pyrrolidone (PVP) was first

demonstrated by Maurer (1956, 1957). Andersson (1969) showed that

immunogenic paralysis to PVP resulted when animals were given high
doses of PVP (100 to 1000 ug) two months prior to immunization with
optimal immunogenic doses. It has been shown by numerous investigators
that only high molecular weight preparations of PVP induce a measurable
antibody response (Maurer, 1956; Andersson, 1969; Inaba et al., 1978a;
Zimecki & Webb, 1978).

Andersson and Blomgren (1971) demonstrated the T-independent
nature of PVP in experiments with thymectomized, irradiated and bone
marrow reconstituted mice. These findings were supported by those
of Kerbel and Eidinger (1971) and Lake and Reed (1976a). The latter
investigators found that T cell depletion by anti-lymphocyte serum
(ALS) treatment actually enhanced the antibody response to PVP.
More recent studies by Kerbel and Eidinger (1972) have shown that
adult thymectomy induces a significant increase in the ability of
mice to respond to immunization with PVP. These results suggested
the existence of a population of short-lived suppressor T lymphocytes.

Rotter and Trainin (1974) were able to reverse the effect of
adult thymectomy on anti-PVP antibody responses either by thymus
implantation or by a single injection of thymic cells. In addition,
injection of thymic cells suppressed the anti-PVP response of normal
mice. The suppressive cell appeared to be hydrocortisone resistant
and radiosensitive. Mice undergoing a mild graft-versus-host reaction
also displayed enhanced anti-PVP responses (Rotter & Trainin, 1975).
Again, this enhancement appeared to be due to the elimination of a
T cell population able to regulate the immune response to PVP.

Several investigators have reported studies on the nature of
the immunoregulation of the anti-PVP response. Lake and Reed (1976b)
demonstrated that mice primed by injection with PVP exhibited a
significantly reduced response to later immunization with the PVP
antigen. Zimecki and Webb (1978) showed that maximal suppression
of anti-PVP antibody responses was obtained when mice were primed
with a low molecular weight (10 kd), nonimmunogenic form of PVP (PVP_{10})
four days prior to immunization with a large molecular weight (360 kd),
immunogenic form of PVP (PVP_{360}). These investigators also demonstrated
that low molecular weight preparations of PVP are immunogenic only
in the absence of T cells while high molecular weight preparations
are immunogenic for the intact immune system.

Inaba and coworkers (1978a, 1978b) have extensively characterized
the PVP-induced suppressor cell population. These studies suggest
the existence of two separate populations of T suppressor cells specific
for PVP. The first, termed the "intrinsic" suppressor cell population,
is present in normal mice and functions after a single immunization
with PVP. The intrinsic suppressor cell is thymus dependent, rel-
atively short-lived, hydrocortisone resistant, insensitive to small
dosages of ALS and adherent to nylon wool. The second population is
termed the "extrinsic" suppressor and may be activated after priming
with PVP. The extrinsic suppressor is induced most efficiently by
PVP_{10}. The precursor of the extrinsic suppressor cell is sensitive
to small dosages of ALS, hydrocortisone resistant and thymus-dependent.
The induced extrinsic suppressor itself is sensitive to hydrocortisone,
radiosensitive and nonadherent to nylon wool.

Zimecki and Webb (1976) have attempted to identify the regulatory factors associated with PVP suppression. These investigators have shown that prostaglandin synthetase inhibitors enhance the plaque forming cell response to PVP. This enhancement is seen even in athymic (nu/nu) mice, which suggests that the mediating factor, prostaglandin, is not produced by T cells. More extensive studies have shown that changes in prostaglandin and cyclic nucleotide levels occur after PVP immunization in vivo (Jamieson & Webb, 1977; Zimecki et al., 1980). The variation in these levels is dependent on both the size of the PVP molecule employed and on the influence of T cells in the system. More complete information on the relation of these changes in prostaglandin and cyclic nucleotide levels, and the regulation of the response to PVP, is necessary before definite conclusions can be drawn. It should be pointed out, however, that the current literature suggests that prostaglandin synthesis is an important component of the anti-PVP response.

Although the degree of macrophage dependence in PVP-induced antibody responses has not been clearly elucidated at this time, reports indicate that PVP induces an antibody response even when macrophage function is impaired (Wong & Herscowitz, 1979). In addition, normal PVP responses occur in B cell populations which have been depleted of a large percentage of their macrophages (Zimecki & Webb, 1976; Zimecki et al., 1980). The possibility that low levels of macrophages are necessary for an antibody response to PVP to occur is not ruled out by this data.

The experiments cited here have made it apparent that PVP is an antigen with many interesting properties. These studies suggest that PVP may serve as a model antigen for the investigation of such diverse aspects of the immune system as B cell activation, suppressor cell function and regulation, and accessory cell involvement in T-independent immune responses.

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THE SYNTHESIS AND IMMUNOGENICITY

OF 3-(2,4-DINITROBENZENE SULFONYL)

POLYVINYL PYRROLIDONE

Catherine R. Adams-Burton[1], Lester P.J. Burton[2] and Thomas J. Rogers[1]

[1]Department of Microbiology and [2]Department of Chemistry, Oregon State University, Corvallis, Oregon 97332

II. THE SYNTHESIS AND IMMUNOGENICITY
OF 3-(2,4-DINITROBENZENE SULFONYL) POLYVINYL
PYRROLIDONE

INTRODUCTION

Several investigators, including Inman and Dintzis (1969), Fidler (1975), McMaster et al. (1977) and Peacock et al. (1979), have found it advantageous to develop hapten-carrier conjugates which may be used to study the immune response to T-independent antigens. The synthesis of these conjugates has permitted the study of several antigen systems in which, due to the difficulty of observing anti-carrier antibody responses, previous results were unobtainable. We report here a method which may be used to couple the hapten 2,4-dinitro-phenyl (DNP) to the T-independent antigen, polyvinyl pyrrolidone (PVP).

A number of investigators have demonstrated that preimmunization of mice with a low molecular weight (10 kd), non-immunogenic prepara-tion of PVP (PVP_{10}) is able to activate a suppressor cell population(s) capable of specifically suppressing the response to a later injection with a high molecular weight (360 kd), immunogenic form of PVP (PVP_{360}) (Lake & Reed, 1976b; Inaba et al., 1978a, 1978b; Zimecki & Webb, 1978). These cells appear to be thymus-derived (T) lymphocytes (Inaba et al., 1978a, 1978b) and may be regulated by prostaglandin synthesis (Zimecki & Webb, 1976; Jamieson & Webb, 1977; Zimecki et al., 1980). It is our belief that PVP offers an ideal opportunity for the further elucidation of the nature of suppressor cell regulation. Since measur-ing anti-PVP antibody responses is a rather tedious procedure due to

the small size of the plaques produced, we have attempted to conjugate the PVP with the DNP hapten. In this way, the antibody response may be more conveniently assessed by measuring the anti-DNP plaque forming cell responses.

Our studies with DNP-PVP indicate that it is immunogenic in mice both in vivo and in vitro, and retains the T-independent nature of PVP with similar dose-response kinetics. Although the DNP-PVP is not highly immunogenic, it does induce a response which compares well with the anti-PVP antibody responses reported by others (Lake & Reed, 1976a, 1976b; Zimecki & Webb, 1976, 1978; Wong & Herscowitz, 1979; Zimecki et al., 1980). Preimmunization with the low molecular weight PVP preparations induces a significant suppression of the antibody response to DNP-PVP. Priming with PVP_{10} reduces both anti-DNP and anti-PVP antibody-producing cells. The PVP_{10}-induced suppression is apparently carrier-specific however, since other DNP-carrier conjugates are not affected. We propose that the synthesis of this conjugate offers an excellent opportunity for further analysis of the PVP-induced suppressor system.

MATERIALS AND METHODS

Mice

Male BALB/c mice were obtained from Simonsen Laboratories (Gilroy, CA) and the Fred Hutchinson Cancer Research Center (Seattle, WA). Athymic male BALB/c (nu/nu) mice were obtained from the Fred Hutchinson Cancer Research Center. All experiments were conducted with mice between the ages of eight and twelve weeks.

Materials

Polyvinyl pyrrolidone with molecular weights of 360 kd (PVP_{360}) and 10 kd (PVP_{10}) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,4-dinitrobenzene sulfonyl chloride was obtained from Aldrich Chemical Co. (Milwaukee, WI). 2,4,6-trinitrobenzene sulfonic acid (TNP), used in the plaque forming cell (PFC) assay, was obtained from Sigma.

Dimethylformamide (DMF), diisopropylamine and n-butyl lithium were obtained from Aldrich. Dimethylformamide was dried over $MgSO_4$, distilled and stored over Type 4A molecular sieves (J.T. Baker Chemical Co.; Phillipsburg, NJ). Diisopropylamine was distilled over CaH_2 and stored over molecular sieves as just described.

Sheep erythrocytes (SRBC) used in the plaque forming cell assays were obtained fresh from one sheep, and were aged five to fourteen days before use. Fresh guinea pig serum, adsorbed against SRBC, was used as a source of complement (Rockland Co.; Rockland, MD).

Chemical Analysis

The extent of coupling with DNP was determined spectrophoto-
metrically using a Beckman Model 35 Spectrophotometer at 255 nm.
The amount of hapten coupled to the PVP was estimated using the molar
extinction coefficient calculated for free 2,4-dinitrobenzene sulfonic
acid at 255 nm. Due to adsorption at 255 nm, a correction factor
was determined for the contribution of PVP to the optical densities
obtained with DNP-PVP conjugates. For DNP_{24}-PVP_{360} this was approx-
imately nine percent. Addition of hapten does not appear to signif-
icantly alter the underlying absorption of the PVP or change the
maximum adsorbance spectra of DNP.

Preparation of 3-(2,4-dinitrobenzene sulfonyl) polyvinyl pyrrolidone

A dried 100 ml round bottom flask with magnetic stirring bar
was filled with argon and covered with a rubber septum. To produce
a conjugate with 24 DNP per PVP molecule, 0.5 g PVP_{360} was dissolved
in 40 ml dried DMF in the flask. A 20 gauge needle attached to an
argon filled balloon was inserted through the septum to maintain
an inert atmosphere. This was placed in an ice bath and allowed to
cool ten to fifteen minutes while stirring. The flask was maintained
under a positive pressure of argon throughout the reaction. A 0.2
ml portion of a 1.6 M solution of n-butyl lithium in hexane was added
dropwise. Stirring was continued for 30 minutes. A 38 mg solution
of 2,4-dinitrobenzene sulfonyl chloride in five ml DMF was added
and allowed to stir 24 hours at room temperature. The procedure

from the addition of n-butyl lithium to the final 24 hour stirring
was repeated an additional two times.

Slightly different conditions were used to produce conjugates
with 0.03 to 10 DNP per PVP. Diisopropylamine and n-butyl lithium
were added to five ml DMF in a dried 50 ml round bottom flask under
argon and stirred ten minutes. This solution was added dropwise
to one gram PVP_{360} as previously described. Stirring was allowed
to continue 30 minutes. 2,4-dinitrobenzene sulfonyl chloride was
added and stirring was continued for 90 minutes. Data showed that var-
iations in the method of preparation of these conjugates resulted
in differences in extent of coupling as indicated in Table 1.

All reactions were quenched with ten ml distilled water and
solvent was removed by distilling under vacumn. The conjugates were
resuspended in distilled water, extensively dialyzed against several
changes of distilled water over a period of two weeks and column
purified by passage over a Sephadex G-100 column in distilled water.
Water was removed from the purified conjugates by lyophilyzation.
The conjugates were stored in light-proof desiccators. The number
of DNP groups per PVP molecule was determined by U.V. using a molar
extinction coefficient of 1.2×10^4 as previously described.

Immunizations

Mice were immunized intravenously with 0.25 ug DNP-PVP in sterile
saline except where indicated. Preparations of DNP-PVP were sterilized
by exposure to ultraviolet light. When millipore membrane filtration

was attempted, loss of the antigen due to membrane binding resulted.

Preparation of cell suspensions for use in cell cultures

Mice were sacrificed by cervical dislocation. Spleens were removed asceptically and single-cell suspensions were prepared as described by Rogers & Balish (1978) with slight modifications. Cells were harvested, washed two times in minimum essential medium (MEM; Grand Island Biological Corp. (GIBCO), Grand Island, NY) containing ten percent fetal calf serum (FCS; GIBCO) and adjusted to the required cell concentration in culture medium.

Cell cultures

Cells were cultured in micro Mishell-Dutton cultures as described by Kappler (1974) and modified by Tittle and Rittenberg (1978). The following culture medium was used throughout: 86 ml suspension MEM (Microbiological Associates (MBA), Walkersville, MD) supplemented with one ml 100X non-essential amino acids (MBA), one ml 100 mM sodium pyruvate (MBA), 0.1 ml 50 mg/ml gentamicin (Schering Corp., Kenilworth, NJ), one ml 200 mM L-glutamine (GIBCO), ten ml FCS, one ml 2-mercaptoethanol (1:20 dilution of a 0.1 M solution in distilled water; SIGMA) and one ml of an adenosine, uridine, cytosine and guanosine solution (one gram each/liter MEM; SIGMA).

Appropriate concentrations of DNP-PVP in a volume of 0.05 ml were placed in the wells of a microtiter tissue culture plate (Falcon Plastics). After ultraviolet sterilization, plates were placed in

a sealed box and incubated 30 minutes at 37 C in an atmosphere of

83% N_2, 7% O_2 and 10% CO_2 to stabilize the pH at 7.0 to 7.3. A 0.05

ml volume containing 1.0 to 1.1 X 10^6 splenocytes was added to each

well and the plates incubated at 37 C in the atmosphere just described

for a period of four or five days. Twenty-four hours after initiation

of incubation, cells were fed 0.05 ml of nutritional cocktail prepared

as follows: A stock solution consisting of 140 ml MEM containing

0.14 ml 50 mg/ml gentamicin, 20 ml 50X essential amino acids (MBA),

ten ml 100X non-essential amino acids, ten ml 200 mg/ml dextrose

(GIBCO) and ten ml 200 mM L-glutamine was adjusted to a pH of 7.2

with one N NaOH, and 30 ml 7.5% $NaHCO_3$ (GIBCO) was added. To five

ml stock solution, 7.5 ml FCS and 0.33 ml nucleoside solution were

added, the mixture was millipore membrane filtered and used to supple-

ment one microtiter plate.

The contents of eight wells were pooled for assay as one culture.

Several replicate cultures for a given experimental point were set

up and two to four assays were conducted with each culture.

Spleen cell priming with PVP_{10}

Splenocytes were suspended in cell culture media at a concentra-

tion of 1.5 X 10^7 cells/ml. Ten ml volumes of cells were cultured

in glass petri plates in the presence of 10^{-4} ug PVP_{10} per ml and

an atmosphere of 83% N_2, 7% O_2 and 10% CO_2. After 24 hours of culture,

the cells were harvested by gentle agitation and by scraping the dish

with a rubber policeman. The cell suspensions from two or three

dishes were pooled, washed twice with MEM supplemented with ten percent

FCS, and suspended in culture media at appropriate concentrations.

Detection of the anti-TNP response

Experiments performed in vivo showed that anti-TNP responses reached a peak at five days post-injection. A single-cell suspension from injected mice was prepared as previously described except that washing and final suspension were done in MEM without FCS. Four assays were performed for each group per experiment and are expressed as one experimental point. In vitro cultures were harvested on the fourth or fifth day of culture as indicated.

All cell preparations were assayed for anti-TNP plaque forming cells (TNP-PFC) according to the method of Cunningham and Szenberg (1968). Briefly, 100 ul spleen cells, 20 ul of a 25% solution of TNP-sheep erythrocytes (TNP-SRBC), 20 ul complement diluted 1:2 and 25 ul MEM were mixed, placed in Cunningham chambers, sealed with a parafin-vaseline mixture and incubated 30 minutes at 37 C. Plaques were counted at 150X magnification. Conjugation of TNP-SRBC was done according to the method of Rittenberg and Pratt (1969). Only the direct (IgM) plaque forming cell response was assessed.

RESULTS

Immunogenicity of DNP-PVP$_{360}$ conjugates

BALB/c mice were intravenously immunized with 0.25 ug of a variety of selected DNP-PVP$_{360}$ conjugates and the number of anti-TNP plaque forming cells was assessed (Figure 1). The results indicate that immunogenicity of DNP-PVP$_{360}$ is only achieved with relatively high conjugation ratios of 22 or greater moles of DNP per mole of PVP$_{360}$. The solubility of these molecules is extremely low, and consequently, the synthesis of conjugates with higher DNP to PVP ratios is severely limited. Nevertheless, the DNP$_{24}$-PVP$_{360}$ preparation was sufficiently immunogenic for further analysis and was employed in the following experiments.

Immunogenicity of DNP$_{24}$-PVP$_{360}$ in vivo and in vitro

In order to determine the optimal dosage range of DNP$_{24}$-PVP$_{360}$ in vivo, mice were injected intravenously with various doses of DNP$_{24}$-PVP$_{360}$ ranging from 100 to 0.01 ug. Five days later the number of anti-TNP plaque forming cells was determined (Figure 2). Each result represents the mean $\pm$ standard deviation of three mice. Both PFC/10^6 spleen cells and PFC/spleen are reported. The results clearly show that DNP$_{24}$-PVP$_{360}$ is most immunogenic at a dose of about 0.1 ug. This is similar to the optimal dosage range reported for unconjugated PVP$_{360}$ (Andersson, 1969; Inaba et al., 1978a)

Table 1. Conditions for preparation of DNP-PVP conjugates[a]

Temperature (C)	Reaction Mixture			Moles DNP conjugated per mole PVP
	Diisopropyl-amine (ul)	n-butyl lithium (ul)	DNP (mg)	
22	4	10.5	1.6	0.3
22	20	52.5	4.0	0.03
22	20	52.5	8.0	0.09
22	20	52.5	16	0.56
22	100	104	41	1.4
22	200	210	81	1.7
0[b]	1000	1020	407	8.9
0[b]	3000	3040	1220	10.0
0[c]	0	400	76	22.0
0[d]	0	400	76	24.0

[a]Reactions were carried out with 1.0 g PVP in 40 ml DMF. Varying amounts of lithium-diisopropylamine or n-butyl lithium alone were added and the reaction mixtures stirred for 30 minutes. Varying amounts of DNP in 5 ml DMF were added and the reaction quenched after 90 minutes of stirring.

[b]The stirring times after base and DNP addition were doubled for these two reactions.

[c]The stirring time after DNP addition was extended to 24 hours for this reaction.

[d]The stirring time after DNP addition was extended to 24 hours and the addition of n-butyl lithium and DNP was repeated an additional two times for this conjugate.

Figure 1. Immunogenicity of DNP-PVP$_{360}$ conjugates

The number of anti-TNP plaque forming cells was determined five days after intravenous injection of the various DNP-PVP$_{360}$ conjugates. Each bar represents the mean of at least three mice.
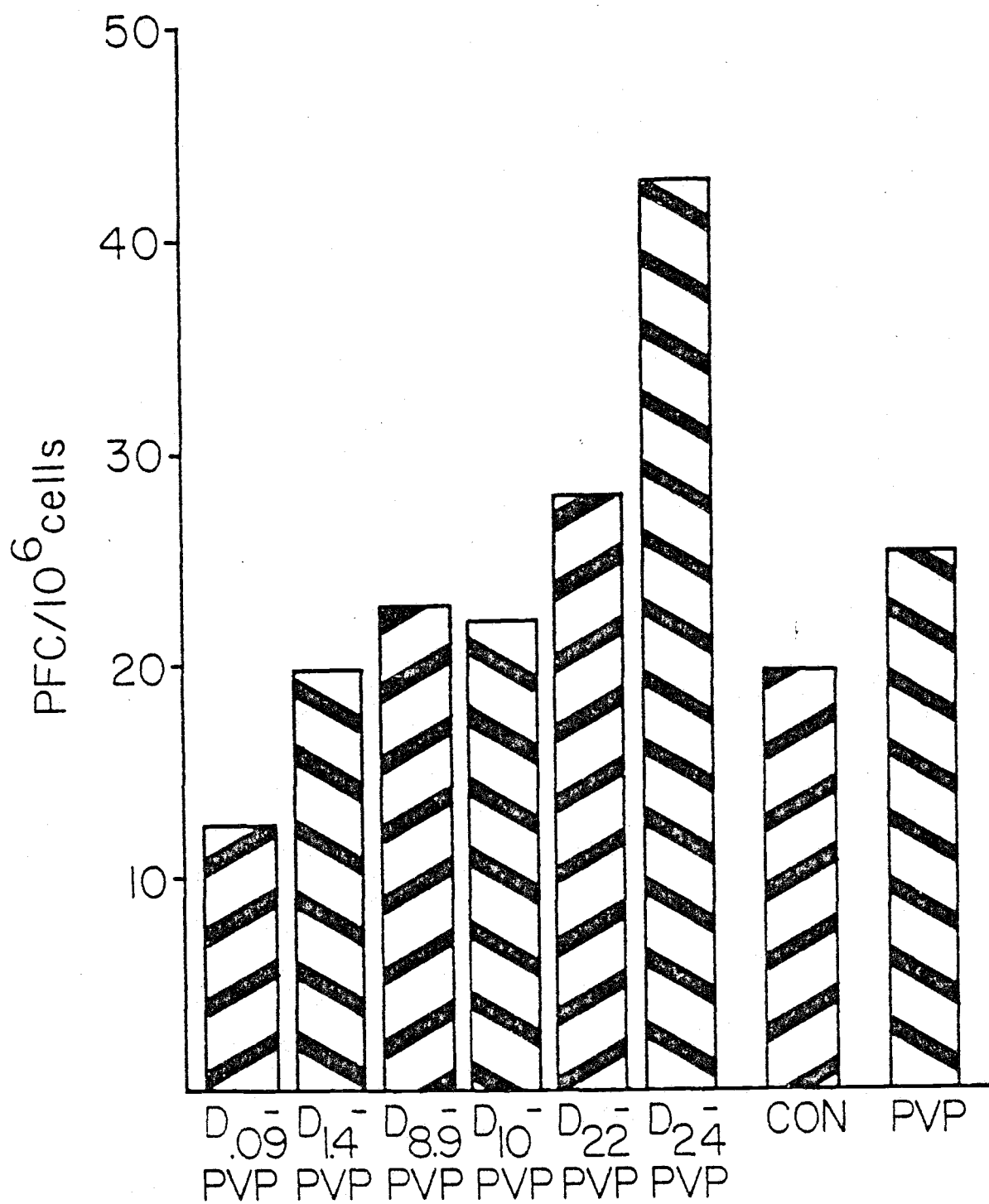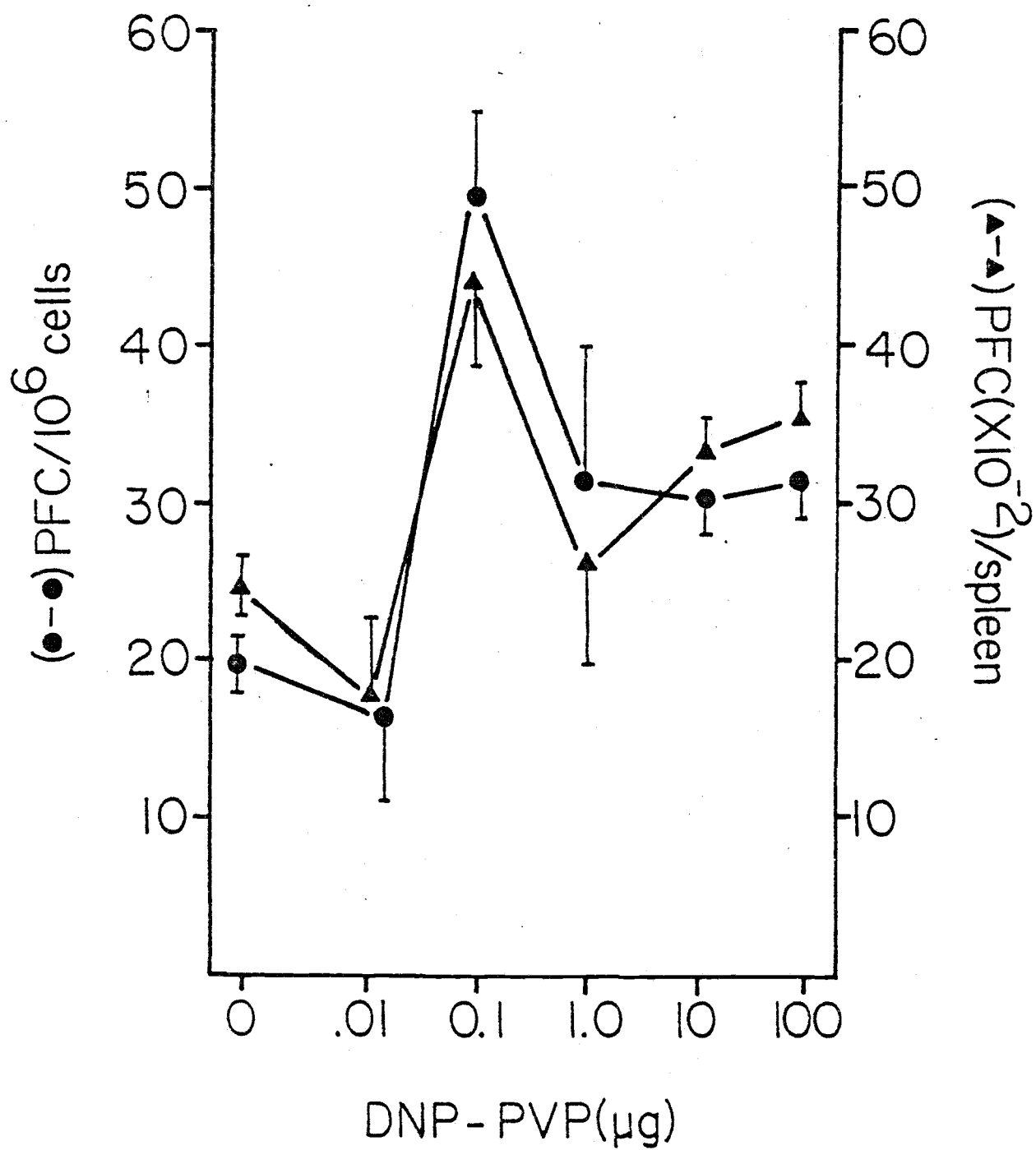
Figure 2. Immunogenicity of DNP_{24}-PVP_{360} _in vivo_

The number of anti-TNP plaque forming cells was determined five days after intravenous immunization with DNP_{24}-PVP_{360}. Each experimental point represents the mean $\pm$ standard deviation (S.D.) of at least three mice.

To determine the immunogenicity of $DNP_{24}-PVP_{360}$ in vitro, 1×10^6 spleen cells/well were cultured in Mishell-Dutton cultures for five days in the presence of varying concentrations of antigen. The development of anti-TNP plaque forming cells was assessed, and the results from two such experiments are presented (Figure 3). The results indicate that, as with reported values for unconjugated PVP_{360} in vitro, a dose of 10^{-5} ug $DNP_{24}-PVP_{360}$ per 10^6 spleen cells is most immunogenic (Zimecki & Webb, 1976, 1978; Zimecki et al., 1980).

Affinity of DNP antibodies

To investigate the affinity of the DNP antibodies produced in response to $DNP_{24}-PVP_{360}$, an experiment was done in which DNP-lysine, at varying concentrations, was added to the plaque assay during incubation. The free DNP-lysine could thus compete with TNP-coupled sheep erythrocytes for DNP antibodies and effectively inhibit the number of plaque forming cells observed. The results (Figure 4) show that, as would be expected, the DNP plaque forming cell response decreases as the DNP-lysine concentration increases. The average relative affinity of the antibody was calculated by determining the point at which 50% inhibition of the plaque forming cell response occurred. This calculated average relative affinity was 9×10^5 M^{-1}. This compares well with affinities of IgM antibodies reported by other investigators for T-dependent and T-independent antigens (Huchet & Feldman, 1973; Inaba et al., 1978a).

Figure 3. Immunogenicity of DNP_{24}-PVP_{360} _in vitro_

The number of anti-TNP plaque forming cells was assessed after spleen cells were cultured for five days in Mishell-Dutton assays in the presence of DNP_{24}-PVP_{360}. Each experimental point represents the mean of at least three cultures $\pm$ standard deviation (S.D.).
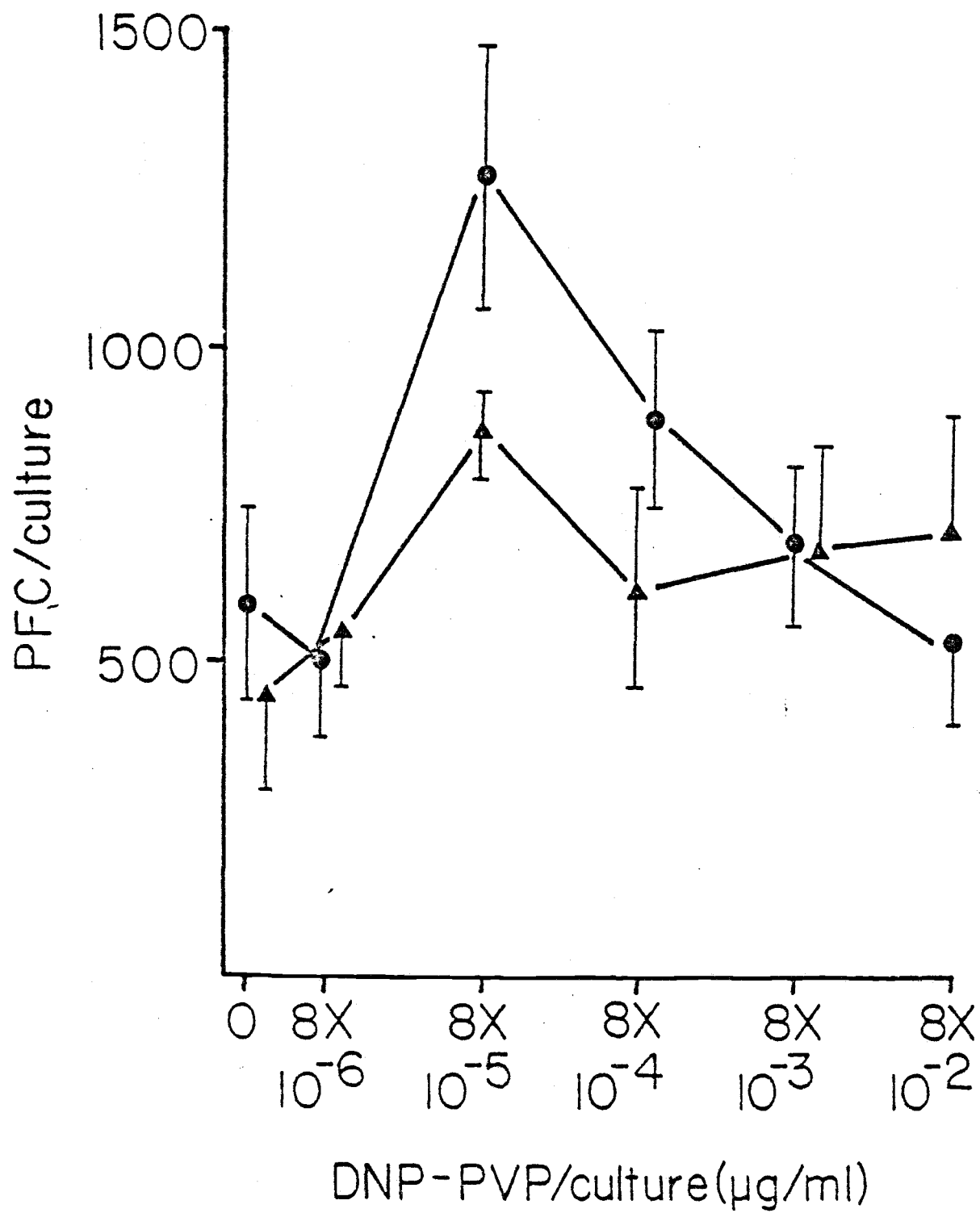
Figure 4. Affinity of DNP antibodies

Five days after intravenous injection of $DNP_{24}-PVP_{360}$, the number
of anti-TNP specific plaque forming cells was determined. Various
concentrations of DNP-lysine were added in the plaque assay as a
competitive inhibitor of DNP-specific plaque forming cells. The
approximate affinity of the anti-DNP antibodies is represented in
the figure by a dotted line. Each experimental point represents the
mean result of at least three mice corrected for background responses
to unconjugated PVP_{360}.
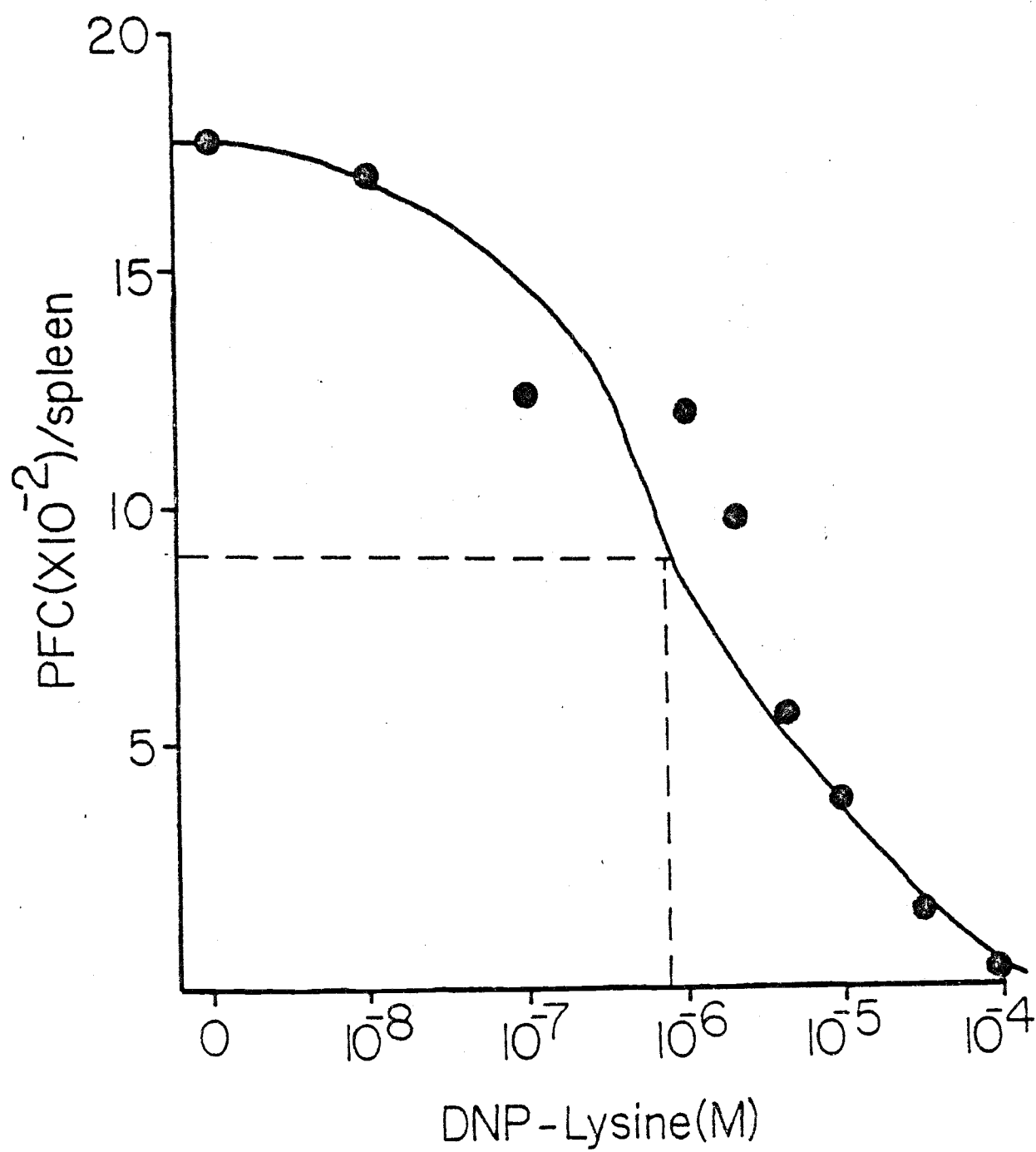
Immunogenicity of DNP_{24}-PVP_{360} in athymic mice

Polyvinyl pyrrolidone is a T-independent antigen (Andersson & Blomgren, 1971) and, since it was possible that the conjugation technique altered this characteristic, we felt it necessary to conduct tests on the T-independent nature of DNP_{24}-PVP_{360}. Congenitally athymic BALB/c (nu/nu) mice were immunized with either 0.025, 0.25 or 2.5 ug DNP_{24}-PVP_{360} or 0.25 ug PVP_{360}. After five days, the number of anti-TNP plaque forming cells was determined (Table 2). The results clearly show that mice deficient in T lymphocyte function manifest a significant anti-DNP-PVP response. Although these mice exhibit some difference in the dose response kinetics, the antibody response observed in athymic mice (53.9 PFC/10^6 spleen cells) is at least as great as that observed in normal mice.

Specificity of DNP_{24}-PVP_{360} induced suppression

Work reported by several investigators has previously shown that preimmunization with PVP_{10} or PVP_{360} significantly reduces the response to later injections of PVP_{360} (Lake & Reed, 1976b; Inaba et al., 1978a, 1978b; Zimecki & Webb, 1978). Priming with PVP does not affect the response to other T-dependent and T-independent antigens (Lake & Reed, 1976b; Inaba et al., 1978a). In an effort to learn more about the specificity of the PVP-induced suppression, an experiment was conducted to investigate the effect of preimmunization on the anti-DNP_{24}-PVP_{360} antibody response. The results (Table 3) show the anti-DNP response was significantly reduced in those mice

Table 2. Immunogenicity of DNP_{24}-PVP_{360} in athymic mice[a]

Immunizations (ug DNP_{24}-PVP_{360})	$PFC/10^6 \pm$ S.D.[b]	PFC/spleen $\pm$ S.D.[b]
0.025	53.9 ± 6.2	7390 ± 856
0.25	26.9 ± 3.3	5175 ± 640
2.5	53.2 ± 11.1	2875 ± 598
0	28.4 ± 3.0	3600 ± 707
0.25 ug PVP_{360}	35.3 ± 6.9	4825 ± 519

[a]The number of anti-TNP plaque forming cells was determined five days after intravenous immunization. Each experimental point represents the mean ± standard deviation of at least three athymic (nu/nu) mice.

[b]S.D. is the standard deviation.

primed with PVP_{10} (42% suppression) or $DNP_{24}-PVP_{360}$ (40% suppression).
No suppression of the anti-DNP response occurred when preimmunization
was followed by immunization with TNP-KLH, indicating that PVP-induced
suppression is carrier (PVP) specific.

Induction of suppression to $DNP_{24}-PVP_{360}$ in vitro

Further investigations of the DNP-PVP suppression are best conducted
in vitro since these methods eliminate many unidentifiable variables
present in vivo. It was considered necessary, therefore, to test
the feasibility of $DNP_{24}-PVP_{360}$ suppressor induction in vitro. Cells
which had been preincubated in the presence or absence of PVP_{10} were
mixed with freshly prepared splenocytes in various ratios (indicated
in Table 4). After four days of incubation in Mishell-Dutton cultures,
the number of anti-TNP plaque forming cells was assessed. In vitro
priming with PVP_{10} significantly reduced the anti-DNP antibody response.
This suppression was not seen with cells preincubated in the absence
of antigen or with cells which had not been preincubated. In addition,
significant suppression was observed when small numbers of primed
cells were mixed and incubated with unprimed cells. A mixture con-
taining 75% untreated cells and 25% PVP_{10} primed cells led to a 49%
suppression relative to untreated cell responses while a 90% untreated
cell, 10% primed cell mixture manifested a 23% reduction in plaque
forming numbers. In both cases, the degree of suppression observed
was too great to be explained by simple dilution of plaque numbers.
The data suggest, therefore, that PVP_{10} priming in vitro generates

Table 3. Specificity of PVP-induced suppression[a]

Group	Priming (ug)	Immunizations (ug)	PFC/10^6 $\pm$ S.D.[c]	PFC/spleen $\pm$ S.D.[c]	percent suppression[d]
A	None	0.25 DNP_{24}-PVP_{360}	33.9 $\pm$ 9.1	3025 $\pm$ 813	
B	None	100 TNP-KLH	34.8 $\pm$ 3.8	N.D.[e]	
C	0.25 DNP_{24}-PVP_{360}	0.25 DNP_{24}-PVP_{360}	20.3 $\pm$ 3.4	2087 $\pm$ 347	40
D	0.25 PVP_{10}	0.25 DNP_{24}-PVP_{360}	19.6 $\pm$ 1.1	1967 $\pm$ 115	42
E	0.25 DNP_{24}-PVP_{360}	100 TNP-KLH	35.7 $\pm$ 1.9	3200 $\pm$ 173	0

[a] The anti-DNP plaque forming response was assessed five days after intravenous immunization with PVP_{360} or intraperitoneal immunization with TNP-KLH. Each experimental point represents the mean $\pm$ standard deviation of at least three mice.

[b] Mice were primed intravenously four days before PVP_{360} or TNP-KLH immunization.

[c] S.D. is the standard deviation.

[d] The percent suppression relative to mice receiving DNP_{24}-PVP_{360} or TNP-KLH was determined.

[e] N.D. means not determined.

an active suppressor cell population which is capable of inhibiting

DNP-specific antibody responses.

Table 4. Induction of suppression to DNP_{24}-PVP_{360} <u>in vitro</u>[a]

Untreated spleen cells (%)[b]	Unprimed spleen cells (%)[c]	Primed spleen cells (%)[d]	PFC/culture	percent suppression[e]
100	0	0	2445	
0	100	0	2700	0
0	0	100	1410	42
90	10	0	2265	N.S.[f]
75	25	0	2295	N.S.[f]
90	0	10	1890	23
75	0	25	1245	49

[a]The number of anti-TNP plaque forming cells was determined after four days incubation in the presence of DNP_{24}-PVP_{360}. Background TNP plaques for each group was as follows: untreated, 1200 PFC/culture; unprimed, 1650 PFC/culture; primed, 2130 PFC/culture. Each experimental point represents the mean of replicate cultures.

[b]Untreated spleen cells were prepared on the day of Mishell-Dutton culture.

[c]Unprimed spleen cells were incubated for 24 hours in the absence of any antigen before Mishell-Dutton culture.

[d]Primed spleen cells were incubated for 24 hours in the presence of PVP_{10} before Mishell-Dutton culture.

[e]The percent suppression relative to untreated cells receiving DNP_{24}-PVP_{360} was determined.

[f]N.S. means not significant.

DISCUSSION

Polyvinyl pyrrolidone has been a valuable model antigen for the study of diverse aspects of the immune response. However, quantitation of the PVP plaque forming cell response can be tedious because of the small plaque size. This is particularly true for plaque forming cell responses in Mishell-Dutton cultures. Several investigators have avoided the technical difficulty of in vitro cultures by using irradiated, thymectomized mice reconstituted with bone marrow cells or bone marrow plus thymus cells (Rotter & Trainin, 1974; Inaba et al., 1978a, 1978b). This method is extremely tedious, time-consuming and expensive. We have circumvented these problems through the development of the DNP-PVP conjugate. Plaques which develop in response to TNP-coupled sheep erythrocytes are quite large and easily detected even with in vitro immunization.

Our experiments indicate that a relatively high hapten to carrier ratio is necessary in order to induce a significant anti-DNP response in mice. Conjugates with less than ten DNP per PVP were non-immunogenic, $DNP_{22}-PVP_{360}$ led to a slight response, and a moderate DNP response was observed with $DNP_{24}-PVP_{360}$. This phenomenon is not restricted to DNP-PVP. Investigators working with DNP-ficoll (Mosier et al., 1974), DNP-polyethylene oxide (Peacock et al., 1979) and TNP-polyacrylamide beads (Feldmann et al., 1974) have found that maximal antibody responses occur at a hapten to carrier ratio of approximately 20 to 40 : 1.

In comparison to DNP-mitogen or other DNP-T-independent conjugates, the magnitude of the anti-DNP response to DNP-PVP is not extremely high. The anti-DNP plaque forming response to DNP-PVP is, however, similar to or greater than the anti-PVP plaque forming cell response reported by several investigators (Rotter & Trainin, 1974; Lake & Reed, 1976b; Muraoka et al., 1977; Zimecki & Webb, 1978; Zimecki et al., 1980).

As a part of our analysis of the immune response to DNP-PVP, we attempted to determine the average relative affinity of the anti-DNP antibody by a plaque inhibition assay. Plaque forming cells producing antibodies with high affinity are inhibited by a lower concentration of free hapten than are lymphocytes producing antibody with low affinity. Immunoglobulin M antibody responses traditionally have a lower affinity than IgG antibody responses. Our calculated affinity for the anti-DNP response to DNP-PVP, $9 \times 10^5 \text{ M}^{-1}$, is comparable to other, primarily IgM, antibody responses (Huchet & Feldman, 1973; Inaba et al., 1978a).

Several methods have been employed to demonstrate whether a particular antigen is T-independent. Some of these methods include the use of irradiated, thymectomized and bone marrow cell reconstituted mice (Andersson & Blomgren, 1971; Inaba et al., 1978), treatment with anti-lymphocyte serum in vivo or in vitro (Kerbel & Eidinger, 1971; Mosier et al., 1974; Peacock et al., 1978), congenitally athymic (nu/nu) mice (Mosier et al., 1974) or combinations of these techniques. Experiments reported here with DNP-PVP vaccinated congenitally athymic

mice strongly indicate the conjugate has retained the T-independent nature of PVP.

Suppression of the PVP response, easily induced by priming with PVP_{10} prior to PVP_{360} immunization, is one of the more interesting characteristics of the anti-PVP response. This suppression has been shown to be controlled by a population(s) of T cells which are anti-thymocyte serum and hydrocortisone sensitive, disappear after adult thymectomy and are specific for PVP (Inaba et al., 1978a, 1978b). We have observed PVP_{10}-induced suppression of anti-DNP plaque forming cell responses with DNP-PVP immunization. This suppression appears to be carrier modulated as evidenced by our inability to suppress TNP-KLH responses after induction of the PVP suppressor cell.

In summary, DNP-PVP has retained the characteristics of PVP_{360}, in so far as it is T-independent, has similar dose response kinetics and is sensitive to PVP_{10}-induced, carrier-specific suppression. Further analysis of the regulation of the anti-PVP response should provide important information on the nature of T cell mediated immunoregulation in general. We feel the production of the DNP-PVP conjugate offers an excellent tool for these studies.

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THE EFFECT OF PROSTAGLANDIN SYNTHETASE INHIBITION

ON INDUCTION OF SUPPRESSION TO

POLYVINYL PYRROLIDONE IN VIVO

Catherine R. Adams-Burton and Thomas J. Rogers

Department of Microbiology, Oregon State University, Corvallis, Oregon 97332

III. THE EFFECT OF PROSTAGLANDIN SYNTHETASE INHIBITION
ON INDUCTION OF SUPPRESSION TO
POLYVINYL PYRROLIDONE IN VIVO

INTRODUCTION

It has been suggested that prostaglandins play a regulatory
role in the immune responses to several antigens (Webb & Osheroff,
1976; Zimecki & Webb, 1976; Webb & Nowowiejski, 1977; Zimecki et al.,
1980). This appears to be particularly true for the response to the
T-independent antigen, polyvinyl pyrrolidone (PVP). Several inves-
tigators have demonstrated the presence of suppressor cells functioning
in the response to PVP (Kerbel & Eidinger, 1972; Rotter & Trainin, 1974;
Inaba et al., 1978a, 1978b; Zimecki & Webb, 1978). Two separate
populations of these cells have been described; one readily activated
by a single injection of an immunogenic, high molecular weight form
of PVP (360 kd; PVP_{360}) and another which may be induced only after
"priming" with a non-immunogenic, low molecular weight preparation
of PVP (10 kd; PVP_{10}) before PVP_{360} immunization. The former popula-
tion has been termed the intrinsic suppressor cell and the latter
the extrinsic suppressor cell (Inaba et al., 1978a, 1978b). Thus,
the anti-PVP response would appear to offer an excellent model system
for studying the relationship between prostaglandins and suppressor
cell regulation. Zimecki and Webb (1976) have investigated and
postulated a regulatory function for prostaglandins in the functioning
of the intrinsic suppressor cell population. Our experiments were
designed to investigate the possible role of prostaglandins in reg-
ulating the induced or extrinsic suppressor cell species.

MATERIALS AND METHODS

Mice

Male BALB/c mice were obtained from Simonsen Laboratories (Gilroy, CA) and the Fred Hutchinson Cancer Research Center (Seattle, WA). They were maintained in our animal facilities and used at eight to twelve weeks of age.

Immunizations

Mice were immunized intravenously with 0.25 ug polyvinyl pyrrolidone at molecular weights of 360 kd or 10 kd (Sigma Chemical Co., St. Louis, MO). Mice receiving prostaglandin synthetase inhibitors (PGSI) were injected intraperitoneally twice over an eight to ten hour period with either 0.15 mg indomethacin (Sigma) or 0.6 mg ($\pm$)-6-chloro-α-methylcarbazole-2-acetic acid (Ro-20-5720; Hoffman-LaRoche, Nutley, NJ).

Binding of antigen to SRBC

To couple PVP to sheep erythrocytes (SRBC), 20 ml of a 3% SRBC suspension in saline was mixed with 20 ml of a tannic acid solution (0.05 mg tannic acid per ml saline) and incubated at 37 C for 15 minutes. Cells were washed several times in saline, resuspended at 3% and an equal volume of PVP_{10} (0.5 mg/ml) was added, mixed and allowed to stand for 15 minutes at 37 C. Cells were washed several times and resuspended at 25% in modified barbital buffer (pH 7.3; Garvey et al., 1977).

Detection of the anti-PVP response

Mice were sacrificed by cervical dislocation. Spleens were
removed and single cell suspensions were prepared as described by
Rogers and Balish (1978) with slight modifications. Cells were har-
vested, washed two times in minimum essential medium (MEM; Grand
Island Biological Corp., Grand Island, NY) and adjusted to the re-
quired cell concentration in MEM. All cell preparations were assayed
for anti-PVP plaque forming cells (PVP-PFC) according to the method
of Cunningham and Szenberg (1968). Briefly, 100 ul spleen cells,
20 ul of a 25% PVP-sheep erythrocytes (PVP-SRBC) suspension, 20 ul
fresh guinea pig serum (diluted 1:2) and 25 ul MEM were mixed, placed
in Cunningham plaque assay chambers, sealed with a parafin-vaseline
mixture and incubated 90 to 120 minutes at 37 C. Plaques were counted
at 150X magnification. Three to four assays were performed on each
group per experiment and are expressed as one experimental point.
All data was reported in terms of direct (IgM) plaque forming cells.

RESULTS AND DISCUSSION

We examined the effect of prostaglandin synthetase inhibition on induced suppression of PVP_{360} responses by treating mice with either indomethacin, an irreversible prostaglandin synthetase inhibitor, or $(\pm)$-6-chloro-α-methyl-carbazole-2-acetic acid (Ro-20-5720). Ro-20-5720, a competitive, reversible inhibitor of prostaglandin synthetase, has been shown to block the consumption of oxygen in the conversion of arachodonate to its endoperoxide intermediate (Grant et al., 1975). To date, this is the only known action of this compound on any metabolic function.

When indomethacin was used, groups (consisting of three mice each) received two injections over a 12 hour period at the time of PVP_{10} injection (4 days before PVP_{360}) or three days after PVP_{10} injection (one day before PVP_{360}). In addition, control groups received identical indomethacin treatments but no PVP_{10} and were assayed along with groups receiving only PVP_{10} and PVP_{360}, or PVP_{360} alone. Five days after final PVP_{360} immunization, all mice were assayed for the presence of PVP plaque forming cells. The data presented in Table 1 show that, as expected, mice primed with PVP_{10} exhibited a suppressed anti-PVP response when challenged four days later with PVP_{360}. The PVP_{10}-induced suppression was found, however, to be unaffected by indomethacin. Indeed, the indomethacin alone, given at the time of (and in place of) the PVP_{10} priming, induced significant suppression of the anti-PVP response.

Table 1. The effect of indomethacin on the antibody response to PVP_{360} in vivo[a]

Group	PVP_{10} Priming[b]	Time of indomethacin treatment (relative to PVP_{360})[c]	$PFC/10^6$ $\pm$ S.D.[d]	PFC/spleen $\pm$ S.D.[d]	percent suppression[e]
A	−	None	203.7 $\pm$ 12.2	24,413 $\pm$ 1803	
B	+	None	102.2 $\pm$ 3.3	12,813 $\pm$ 442	50
C	+	Four days before	121.8 $\pm$ 14.5	13,517 $\pm$ 1390	40
D	+	One day before	151.0 $\pm$ 35.0	15,850 $\pm$ 2993	26
E	−	Four days before	101.7 $\pm$ 6.2	13,033 $\pm$ 660	50
F	−	One day before	102.9 $\pm$ 29.1	13,738 $\pm$ 3660	49

[a]The anti-PVP plaque forming response was assessed five days after intravenous immunization with PVP_{360}. Each experimental point represents the mean $\pm$ standard deviation of three mice assayed a minimum of three times.

[b]Mice were primed with 0.25 ug PVP_{10} intravenously four days before PVP_{360} immunization.

[c]Mice treated with indomethacin received two 0.15 mg intraperitoneal injections over a ten to twelve hour period.

[d]S.D. is the standard deviation.

[e]The percent suppression relative to mice receiving PVP_{360} alone was determined.

When Ro-20-5720 was used as the prostaglandin synthetase inhibitor (Table 2), mice (four groups, each consisting of four mice) received two injections separated by an eight to ten hour period either one day before, at the time of, or one or two days after PVP_{10} injection (five to two days before PVP_{360} immunization). Again, control groups were assayed alone with experimental groups. Consistent with the results obtained from mice treated with indomethacin, Ro-20-5720 did not appear to alter the ability of PVP_{10} to suppress the anti-PVP response after challenge with PVP_{360}. In addition, control groups treated with Ro-20-5720 but not PVP_{10} displayed very significant suppression relative to the PVP_{360} control. The results of these two experiments indicate that when prostaglandin synthesis is inhibited in vivo, no affect on the functioning of the PVP_{10}-induced extrinsic suppressor cell population is observed. However, it also appears that prostaglandin synthesis inhibition alone is capable of mimicking the activity of the extrinsic suppressor cell population.

While investigating the effect of inhibitors of prostaglandin synthesis on the in vitro immune responses to T-independent antigens, Zimecki and Webb (1976) treated spleen cell cultures with indomethacin or Ro-20-5720 at varying times relative to PVP_{360} immunization. The main finding from these experiments was that addition of prostaglandin synthetase inhibitors at a four to eight hour period after PVP_{360} addition led to a significant enhancement of plaque forming cell responses. This was true even when spleen cell cultures consisted of highly purified B lymphocytes, presumably lacking the intrinsic suppressor cell population. Thus, it was postulated that PVP-stimulated

Table 2. The effect of Ro-20-5720 on the antibody response to PVP_{360} _in vivo_[a]

Group	PVP_{10} Priming[b]	Time of Ro-20-5720 treatment (relative to PVP_{360})[c]	$PFC/10^6$ $\pm$ S.D.[d]	PFC/spleen $\pm$ S.D.[d]	percent suppression[e]
A	−	None	415.0 ± 16.6	75,100 ± 2996	
B	+	None	270.5 ± 16.3	36,250 ± 2181	35
C	+	Five days before	259.2 ± 4.8	31,100 ± 577	38
D	+	Four days before	256.5 ± 28.3	35,650 ± 3961	38
E	+	Three days before	136.9 ± 23.6	17,250 ± 2973	67
F	+	Two days before	147.8 ± 21.8	22,900 ± 3380	64
G	−	Four days before	285.3 ± 45.6	47,650 ± 7615	31
H	−	Three days before	180.6 ± 17.2	24,200 ± 2304	56
I	−	Two days before	192.1 ± 21.8	23,050 ± 2615	54

[a] The anti-PVP plaque forming response was assessed five days after intravenous immunization with PVP_{360}. Each experimental point represents the mean ± standard deviation of four mice assayed a minimum of three times.

[b] Mice were primed with 0.25 ug PVP_{10} intravenously four days before PVP_{360} immunization.

[c] Mice treated with Ro-20-5720 received two 0.6 mg intraperitoneal injections over an eight to ten hour period.

[d] S.D. is the standard deviation.

[e] The percent suppression relative to mice receiving PVP_{360} alone was determined.

B cells are capable of synthesizing prostaglandins which, in turn, regulate the functioning of such B cells.

Zimecki and Webb (1976) also found that prostaglandin synthetase inhibitors added 24 hours prior to immunization with PVP_{360} inhibited rather than enhanced the plaque forming cell response. This suppression was observed when unfractionated spleen cell cultures were used but not with highly purified B cell populations. This would imply that some cell population(s), present in unfractionated spleen cells but not in purified B cells, was activated by the prostaglandin synthetase inhibitors to suppress the PVP_{360} response. These data are consistent with our findings that prostaglandin synthetase inhibitors are capable of mimicking the extrinsic suppressor cell population. Further in vitro work is necessary before the cell population(s) involved and the mechanism of their regulation can be fully identified.

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APPENDIX: LIST OF ABBREVIATIONS

ALS: anti-lymphocyte serum

ATS: anti-thymocyte serum

C: celsius

C3: third component of the complement system

cAMP: cyclic adenosine monophosphate

con A: concanavalin A

DMF: dimethylformamide

DNP: dinitrophenyl

FCS: fetal calf serum

g: gram

GAT: L-glutamic acid, L-alanine and L-tyrosine

GIBCO: Grand Island Biological Corp.

Ia: associated with the I-region of the histocompatibility locus

IgG: immunoglobulin G

IgM: immunoglobulin M

kd: kilodalton

KLH: keyhole limpet hemocyanin

LPS: lipopolysaccharide

M: molar

MBA: Microbiological Associates

MEM: minimum essential medium

mg: miligram

ml: mililiter

N: normal

nm: nanometer

PAA: polyacrylamide beads

PFC: plaque forming cells

PGE: E series prostaglandins

PGF: F series prostaglandins

PHA: phytohemagglutinin

PITS: prostaglandin-induced-T-cell-derived suppressor

PVP: polyvinyl pyrrolidone

S.D.: standard deviation

SIRS: soluble immune response suppressor

SRBC: sheep erythrocyte

SSS-III: pneumococcal polysaccharide

T-independent: thymic independent

T-lymphocyte or cell: thymic-derived lymphocyte or cell

TNP: trinitrophenyl

ug: microgram

ul: microliter