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-PVP₃₆₀.

We suggest that DNP-PVP₃₆₀ 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 PVP₁₀-induced suppressor cell population. Results indicate that PVP₁₀-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-PVP₃₆₀ conjugate should permit further elucidation of the nature of PVP-induced suppression to be carried out in vitro with isolated cell populations.
A Partial Characterization of the Immune Response to Polyvinyl Pyrrolidone

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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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A PARTIAL CHARACTERIZATION OF THE
IMMUNE RESPONSE TO POLYVINYL PYRROLIDONE

I. REVIEW OF PERTINENT LITERATURE

Lymphocyte populations

One of the principal advances in immunobiology has been the recognition of the differentiation of lymphocytes into two separate populations. 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, transplantation 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 subpopulation 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
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_α, a 35,000 dalton factor and PITS_β, 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, principally 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 lymphocytes 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 lymphocytes in vitro reduce the expression of a number of immune functions (reviewed in Webb et al., 1979). On the other hand, secondary (anamnestic) 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 immunization 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 considerable 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 & Müller, 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 presentation 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 involve 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-independent 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;

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, relatively 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

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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-dinitrophenyl (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 preparation 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 measuring 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 PVP10 reduces both anti-DNP and anti-PVP antibody-producing cells. The PVP10-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<sub>360</sub>) and 10 kd (PVP<sub>10</sub>) 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<sub>4</sub>, distilled and stored over Type 4A molecular sieves (J.T. Baker Chemical Co.; Phillipsburg, NJ). Diisopropylamine was distilled over CaH<sub>2</sub> 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 spectrophotometrically 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 approximately nine percent. Addition of hapten does not appear to significantly alter the underlying absorption of the PVP or change the maximum absorbance 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 PVP360 as previously described. Stirring was allowed to continue 30 minutes. 2,4-dinitrobenzene sulfonyle chloride was added and stirring was continued for 90 minutes. Data showed that variations 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 vacuum. 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 $1.2 \times 10^4$ 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 aseptically 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% N₂, 7% O₂ and 10% CO₂ to stabilize the pH at 7.0 to 7.3. A 0.05 ml volume containing 1.0 to 1.1 X 10⁶ 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% NaHCO₃ (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 supplement 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 PVP₁₀**

Splenocytes were suspended in cell culture media at a concentration of 1.5 X 10⁷ cells/ml. Ten ml volumes of cells were cultured in glass petri plates in the presence of 10⁻⁴ ug PVP₁₀ per ml and an atmosphere of 83% N₂, 7% O₂ and 10% CO₂. 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\textsubscript{360} conjugates

BALB/c mice were intravenously immunized with 0.25 µg of a variety of selected DNP-PVP\textsubscript{360} conjugates and the number of anti-TNP plaque forming cells was assessed (Figure 1). The results indicate that immunogenicity of DNP-PVP\textsubscript{360} is only achieved with relatively high conjugation ratios of 22 or greater moles of DNP per mole of PVP\textsubscript{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\textsubscript{24}-PVP\textsubscript{360} preparation was sufficiently immunogenic for further analysis and was employed in the following experiments.

Immunogenicity of DNP\textsubscript{24}-PVP\textsubscript{360} in vivo and in vitro

In order to determine the optimal dosage range of DNP\textsubscript{24}-PVP\textsubscript{360} in vivo, mice were injected intravenously with various doses of DNP\textsubscript{24}-PVP\textsubscript{360} ranging from 100 to 0.01 µg. Five days later the number of anti-TNP plaque forming cells was determined (Figure 2). Each result represents the mean ± standard deviation of three mice. Both PFC/10\textsuperscript{6} spleen cells and PFC/spleen are reported. The results clearly show that DNP\textsubscript{24}-PVP\textsubscript{360} is most immunogenic at a dose of about 0.1 µg. This is similar to the optimal dosage range reported for unconjugated PVP\textsubscript{360} (Andersson, 1969; Inaba et al., 1978a)
<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Diisopropylamine (ul)</th>
<th>n-butyl lithium (ul)</th>
<th>DNP (mg)</th>
<th>Moles DNP conjugated per mole PVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>4</td>
<td>10.5</td>
<td>1.6</td>
<td>0.3</td>
</tr>
<tr>
<td>22</td>
<td>20</td>
<td>52.5</td>
<td>4.0</td>
<td>0.03</td>
</tr>
<tr>
<td>22</td>
<td>20</td>
<td>52.5</td>
<td>8.0</td>
<td>0.09</td>
</tr>
<tr>
<td>22</td>
<td>20</td>
<td>52.5</td>
<td>16</td>
<td>0.56</td>
</tr>
<tr>
<td>22</td>
<td>100</td>
<td>104</td>
<td>41</td>
<td>1.4</td>
</tr>
<tr>
<td>22</td>
<td>200</td>
<td>210</td>
<td>81</td>
<td>1.7</td>
</tr>
<tr>
<td>b₀</td>
<td>1000</td>
<td>1020</td>
<td>407</td>
<td>8.9</td>
</tr>
<tr>
<td>b₀</td>
<td>3000</td>
<td>3040</td>
<td>1220</td>
<td>10.0</td>
</tr>
<tr>
<td>c₀</td>
<td>0</td>
<td>400</td>
<td>76</td>
<td>22.0</td>
</tr>
<tr>
<td>d₀</td>
<td>0</td>
<td>400</td>
<td>76</td>
<td>24.0</td>
</tr>
</tbody>
</table>

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 ± standard deviation (S.D.) of at least three mice.
To determine the immunogenicity of DNP$_{24}$-PVP$_{360}$ in vitro, $1 \times 10^6$ 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 $9 \times 10^5$ 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 ± 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\textsubscript{24}-PVP\textsubscript{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\textsubscript{24}-PVP\textsubscript{360}. Congenitally athymic BALB/c (nu/nu) mice were immunized with either 0.025, 0.25 or 2.5 µg DNP\textsubscript{24}-PVP\textsubscript{360} or 0.25 µg PVP\textsubscript{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\textsuperscript{6} spleen cells) is at least as great as that observed in normal mice.

**Specificity of DNP\textsubscript{24}-PVP\textsubscript{360} induced suppression**

Work reported by several investigators has previously shown that preimmunization with PVP\textsubscript{10} or PVP\textsubscript{360} significantly reduces the response to later injections of PVP\textsubscript{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\textsubscript{24}-PVP\textsubscript{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$^a$

<table>
<thead>
<tr>
<th>Immunizations (ug DNP$<em>{24}$-PVP$</em>{360}$)</th>
<th>PFC/10$^6$ ± S.D.$^b$</th>
<th>PFC/spleen ± S.D.$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025</td>
<td>53.9 ± 6.2</td>
<td>7390 ± 856</td>
</tr>
<tr>
<td>0.25</td>
<td>26.9 ± 3.3</td>
<td>5175 ± 640</td>
</tr>
<tr>
<td>2.5</td>
<td>53.2 ± 11.1</td>
<td>2875 ± 598</td>
</tr>
<tr>
<td>0</td>
<td>28.4 ± 3.0</td>
<td>3600 ± 707</td>
</tr>
<tr>
<td>0.25 ug PVP$_{360}$</td>
<td>35.3 ± 6.9</td>
<td>4825 ± 519</td>
</tr>
</tbody>
</table>

$^a$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 containing 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

<table>
<thead>
<tr>
<th>Group</th>
<th>Priming (ug)</th>
<th>Immunizations (ug)</th>
<th>PFC/10^6 ± S.D.</th>
<th>PFC/spleen ± S.D.</th>
<th>percent suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>0.25 DNP_24-PVP_360</td>
<td>33.9 ± 9.1</td>
<td>3025 ± 813</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>100 TNP-KLH</td>
<td>34.8 ± 3.8</td>
<td>N.D. e</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.25 DNP_24-PVP_360</td>
<td>0.25 DNP_24-PVP_360</td>
<td>20.3 ± 3.4</td>
<td>2087 ± 347</td>
<td>40</td>
</tr>
<tr>
<td>D</td>
<td>0.25 PVP_10</td>
<td>0.25 DNP_24-PVP_360</td>
<td>19.6 ± 1.1</td>
<td>1967 ± 115</td>
<td>42</td>
</tr>
<tr>
<td>E</td>
<td>0.25 DNP_24-PVP_360</td>
<td>100 TNP-KLH</td>
<td>35.7 ± 1.9</td>
<td>3200 ± 173</td>
<td>0</td>
</tr>
</tbody>
</table>

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 ± 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$_{360}$ \textit{in vitro}\textsuperscript{a}

<table>
<thead>
<tr>
<th>Untreated spleen cells (%)\textsuperscript{b}</th>
<th>Unprimed spleen cells (%)\textsuperscript{c}</th>
<th>Primed spleen cells (%)\textsuperscript{d}</th>
<th>PFC/culture</th>
<th>percent suppression\textsuperscript{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
<td>2445</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>2700</td>
<td>42</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
<td>1410</td>
<td>N.S.\textsuperscript{f}</td>
</tr>
<tr>
<td>90</td>
<td>10</td>
<td>0</td>
<td>2265</td>
<td>N.S.\textsuperscript{f}</td>
</tr>
<tr>
<td>75</td>
<td>25</td>
<td>0</td>
<td>2295</td>
<td></td>
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<tr>
<td>90</td>
<td>0</td>
<td>10</td>
<td>1890</td>
<td>23</td>
</tr>
<tr>
<td>75</td>
<td>0</td>
<td>25</td>
<td>1245</td>
<td>49</td>
</tr>
</tbody>
</table>

\textsuperscript{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 PPC/culture. Each experimental point represents the mean of replicate cultures.

\textsuperscript{b}Untreated spleen cells were prepared on the day of Mishell-Dutton culture.

\textsuperscript{c}Unprimed spleen cells were incubated for 24 hours in the absence of any antigen before Mishell-Dutton culture.

\textsuperscript{d}Primed spleen cells were incubated for 24 hours in the presence of PVP$_{10}$ before Mishell-Dutton culture.

\textsuperscript{e}The percent suppression relative to untreated cells receiving DNP$_{24}$-PVP$_{360}$ was determined.

\textsuperscript{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\textsubscript{22}-PVP\textsubscript{360} led to a slight response, and a moderate DNP response was observed with DNP\textsubscript{24}-PVP\textsubscript{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, $9 \times 10^5$ 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.
REFERENCES


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

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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 investigators 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 population 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 regulating 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 μg 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 (+)-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 PVP₁₀ (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 harvested, washed two times in minimum essential medium (MEM; Grand Island Biological Corp., Grand Island, NY) and adjusted to the required 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 \( \text{PVP}_{360} \) responses by treating mice with either indomethacin, an irreversible prostaglandin synthetase inhibitor, or \((\pm)-6\text{-chloro-} \alpha\text{-methyl-carbazole-2-acetic acid (Ro-20-5720).} \)

Ro-20-5720, a competitive, reversible inhibitor of prostaglandin synthetase, has been shown to block the consumption of oxygen in the conversion of arachidonate 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 \( \text{PVP}_{10} \) injection (4 days before \( \text{PVP}_{360} \)) or three days after \( \text{PVP}_{10} \) injection (one day before \( \text{PVP}_{360} \)). In addition, control groups received identical indomethacin treatments but no \( \text{PVP}_{10} \) and were assayed along with groups receiving only \( \text{PVP}_{10} \) and \( \text{PVP}_{360} \), or \( \text{PVP}_{360} \) alone. Five days after final \( \text{PVP}_{360} \) 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 \( \text{PVP}_{10} \) exhibited a suppressed anti-PVP response when challenged four days later with \( \text{PVP}_{360} \). The \( \text{PVP}_{10} \)-induced suppression was found, however, to be unaffected by indomethacin. Indeed, the indomethacin alone, given at the time of (and in place of) the \( \text{PVP}_{10} \) priming, induced significant suppression of the anti-PVP response.
<table>
<thead>
<tr>
<th>Group</th>
<th>PVP&lt;sub&gt;10&lt;/sub&gt; Priming&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Time of indomethacin treatment (relative to PVP&lt;sub&gt;360&lt;/sub&gt;)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>PFC/10&lt;sup&gt;6&lt;/sup&gt; ± S.D.&lt;sup&gt;d&lt;/sup&gt;</th>
<th>PFC/spleen ± S.D.&lt;sup&gt;d&lt;/sup&gt;</th>
<th>percent suppression&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>None</td>
<td>203.7 ± 12.2</td>
<td>24,413 ± 1803</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>None</td>
<td>102.2 ± 3.3</td>
<td>12,813 ± 442</td>
<td>50</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>Four days before</td>
<td>121.8 ± 14.5</td>
<td>13,517 ± 1390</td>
<td>40</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>One day before</td>
<td>151.0 ± 35.0</td>
<td>15,850 ± 2993</td>
<td>26</td>
</tr>
<tr>
<td>E</td>
<td>-</td>
<td>Four days before</td>
<td>101.7 ± 6.2</td>
<td>13,033 ± 660</td>
<td>50</td>
</tr>
<tr>
<td>F</td>
<td>-</td>
<td>One day before</td>
<td>102.9 ± 29.1</td>
<td>13,738 ± 3660</td>
<td>49</td>
</tr>
</tbody>
</table>

<sup>a</sup>The anti-PVP plaque forming response was assessed five days after intravenous immunization with PVP<sub>360</sub>. Each experimental point represents the mean ± standard deviation of three mice assayed a minimum of three times.

<sup>b</sup>Mice were primed with 0.25 µg PVP<sub>10</sub> intravenously four days before PVP<sub>360</sub> immunization.

<sup>c</sup>Mice treated with indomethacin received two 0.15 mg intraperitoneal injections over a ten to twelve hour period.

<sup>d</sup>S.D. is the standard deviation.

<sup>e</sup>The percent suppression relative to mice receiving PVP<sub>360</sub> 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\textsubscript{10} injection (five to two days before PVP\textsubscript{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\textsubscript{10} to suppress the anti-PVP response after challenge with PVP\textsubscript{360}. In addition, control groups treated with Ro-20-5720 but not PVP\textsubscript{10} displayed very significant suppression relative to the PVP\textsubscript{360} control. The results of these two experiments indicate that when prostaglandin synthesis is inhibited \textit{in vivo}, no affect on the functioning of the PVP\textsubscript{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 \textit{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\textsubscript{360} immunization. The main finding from these experiments was that addition of prostaglandin synthetase inhibitors at a four to eight hour period after PVP\textsubscript{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 PVP360 in vivo\textsuperscript{a}

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

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

\textsuperscript{b}Mice were primed with 0.25 ug PVP\textsubscript{10} intravenously four days before PVP\textsubscript{360} immunization.

\textsuperscript{c}Mice treated with Ro-20-5720 received two 0.6 mg intraperitoneal injections over an eight to ten hour period.

\textsuperscript{d}S.D. is the standard deviation.

\textsuperscript{e}The percent suppression relative to mice receiving PVP\textsubscript{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.
REFERENCES


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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: milliliter
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