Regulation of the immune response to polyvinylpyrrolidone
by Jeffrey Peter Lake

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology
Montana State University

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February, 1977

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. Norman D. Reed, my major advisor, for his guidance and invaluable assistance during the course of these studies. I also thank Dr. Phillip J. Baker for his useful suggestions and the generous donation of type III pneumococcal polysaccharide and antilymphocyte sera.

I wish also to thank the GAF Corporation for donating polyvinylpyrrolidone and Dr. J. T. Ulrich for donating thymosin.

This research was supported by USPHS Research Grants AI-10384 and CA-15322.

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ABSTRACT

The immune response to polyvinylpyrrolidone (PVP), a synthetic polymer, was studied in mice using a localized hemolysis in gel assay. Optimal conditions to detect PVP-specific plaque-forming cells (PFC) were determined and some characteristics of the antibody response to this antigen were defined. To investigate the role of thymus-derived cells (T cells) in the regulation of antibody responses to PVP, the PFC responses of normal and congenitally thymus deficient (nude) mice were compared. Normal and nude mice responded similarly to an optimally immunogenic dose of PVP. Antithymocyte (ATS) or antilymphocyte (ALS) serum treatment of immunized mice caused a 5 to 10-fold increase in the PVP-specific PFC response in normal mice; the response in nude mice was not increased by such treatment. The data suggested that, although T cells are not an absolute requirement in the immune response to this antigen, these cells can regulate the magnitude of the immune response to PVP. Furthermore, depletion of short-lived T cells by adult thymectomy caused enhanced PFC responses to PVP; ATS treatment of adult thymectomized mice resulted in further enhancement. Nude mice implanted with neonatal BALB/c thymus glands (nude-TG) had markedly elevated PFC responses to PVP but not to type III pneumococcal polysaccharide (SSS-III). Nude-TG treated with thymosin had normal numbers of PFC to PVP. These results can be explained by proposing a differential repair of thymus function in nude-TG mice. The activities of helper T cells and T cells amplifying the response to PVP appear to be nearly normal while the activity of cells which would normally exert a negative influence on the magnitude of the PVP response is less than normal.

Prior injection of mice with PVP resulted in the induction of an immunologically unresponsive state detected by measuring the magnitude of the PVP-specific PFC response following the injection of a second, optimally immunogenic dose of PVP. Varying degrees of paralysis were observed in mice primed with PVP doses of 2.5×10^{-5} to 2.5 μg. Paralysis could be detected 1 to 2 days following priming and persisted for at least 20 days. However, 30 days after priming the magnitude of the PVP-specific PFC response was similar to the PFC response of a normal primary response and 60 days after priming the PFC response was 2.2-fold higher than the primary response. Low-dose paralysis was antigen specific because PVP-priming did not alter the magnitude of the PFC response to burro erythrocytes or to SSS-III. The role of suppressor T cells in the induction of low-dose paralysis was investigated. A greater degree of low-dose paralysis was inducible in adult thymectomized and nude-TG mice whereas nude mice could be only partially paralyzed. Attempts to transfer paralysis with cells from PVP-primed

mice were not consistently successful. These results were difficult to interpret making it unfeasible to determine the mechanism of low-dose paralysis.

The results presented here, however, concerning the regulation of immune responses to PVP are consistent with the hypothesis that two functionally distinct types of T cells, suppressor and amplifier cells, act in an opposing manner to regulate the magnitude of the antibody response to PVP by bone marrow derived B cells.

INTRODUCTION

The development of humoral and cell-mediated immunity involves a complex series of cellular and subcellular events (1-3). Cells involved in these events arise from pluripotent hematopoitic stem cells and mature into immunocompetent antigen reactive cells in the thymus or in the mammalian equivalent (bone marrow) of the avian bursa of Fabricus (2). Precursoral lymphocytes develop into clones of immunocompetent cells each of which is committed to respond to a specific antigenic determinant or epitope (4).

Lymphocytes which mature in the thymus (T cells) are responsible for the various cell-mediated immune reactions (2,3). These reactions include the generation of cytotoxic T cells which mediate tumor rejection, allograft rejection, and graft-versus-host reaction and the production of certain lymphokines such as macrophage migration inhibitory factor (MIF), chemotactic factors, lymphotoxins, skin reactive factors, and interferon (3,5). These factors contribute to the inflammatory processes and delayed-type hypersensitivity reactions. T cells may also be important in the resistance and immunity to certain infectious organisms especially intracellular parasites (3,6).

The production of humoral antibody responses to the majority of complex, multideterminant antigens such as heterologous erythrocytes and proteins is dependent on the interactions between at least three functionally distinct cell types, thymus-derived T cells, bone marrow

derived lymphocytes (B cells), and accessory cells usually thought of as adherent cells or macrophages (1,7). The nature of the cell cooperation between these cell types is not presently known, although several useful models have been proposed (7). Ultimately, following the introduction of antigen and the subsequent cellular interactions, specific antigen reactive B cells become activated to differentiate and proliferate into antibody producing cells.

Two classes of antigen have been described according to their dependence on T cell-B cell collaboration for the elicitation of humoral immune responses. As described above, complex multideterminant antigens such as heterologous erythrocytes and proteins require the assistance of T cells to induce antibody producution (1,7). The T cells mediating this function have been labelled helper T cells since they themselves do not produce antibody but rather help B cells to become activated toward a pathway leading to antibody production. Other antigens, however, appear not to require T cells for the induction of immune responses (8), and, accordingly, have been termed thymus-independent (T-independent) antigens. Such antigens are generally large polymers composed of many identical repeating subunits that are poorly metabolized in vivo. Some antigens which have been shown to elicit immune responses in the absence of functional T cells include: pneumococcal polysaccharide (9,10), lipopolysaccharide (10,11),

flagellin (12), Vi antigen (13,14), levan (15), and polyvinylpyrrolidone (11,16,17).

Immune responses have generally been thought of as beneficial, allowing the host to defend against the invasion of infectious agents and perhaps neoplastic cells. However, in some instances immune responses are now recognized to be detrimental to the host. Immune responses have been shown to contribute to the development of a variety of autoimmune diseases, the rejection of beneficial tissue transplants, enhanced tumor growth, anaphylactic reaction, contact hypersensitivities, cell-mediated hypersensitivities, and immune complex diseases (18-20). In view of the beneficial and detrimental aspects of immune responses, it would be extremely useful to be able to manipulate these responses to facilitate protective functions and to discourage harmful effects. To accomplish this goal, it is essential to understand regulatory mechanisms which tend either to initiate immune responses or to limit or terminate these responses.

A great deal of information has been accumulated on the mechanisms that promote antibody production and information is available on the role of antibody or antigen-antibody complexes in feedback inhibition of specific antibody production (21). Recently, Baker (22) and Gershon and Kondo (23) provided evidence that T cells may in some circumstances actively suppress immune responses. Since that time, T cells which negatively regulate immune responses (suppressor T cells),

have been implicated in the development of many diverse phenomena. Suppressor T cells have been shown to contribute to the development and maintenance of some forms of immunological tolerance (24-29). Tolerance mediated by suppressor T cells has been termed infectious tolerance (24), since the injection of spleen cells from a tolerant animal can suppress the response to the appropriate antigen in an otherwise normal recipient. Antigenic competition (24,27) is thought to be due to active suppressive mechanisms mediated by suppressor T cells. The injection of anti-allotype or anti-idiotype serum into mice can result in the inability of these mice to produce antibody of the corresponding allotype or idiotype. Allotype suppression (30) and idiotype suppression (31) were shown to be due to the activity of suppressor T cells. Loss of suppressor T cell function may contribute to the development of some autoimmune diseases (32,33), while evidence has been presented suggesting that the presence of suppressor T cells contributes to some forms of common variable hypogammaglobulinemia (34).

Suppressor T cells may depress humoral antibody responses (24, 25,27) or cell-mediated immune responses (24-27) by either suppressing the activity of other T cells or B cells. Furthermore, the effects of suppressor T cells may be antigen specific or nonspecific. Suppressor T cells may be activated by antigen or by T cell mitogens such as

concanavalin A or the effects of suppressor T cells may be observed following treatments which deplete whole T cell populations.

It is difficult if not impossible to integrate the information presently available concerning suppressor T cells into any coherent model of immunoregulation. Possibly multiple interrelated mechanisms are present, and a simple unified concept may not emerge. In light of these difficulties, the following discussion will be limited to two systems which are pertinent to the study to be presented.

As previously discussed, some antigens (T-independent) do not require the participation of thymus-derived cells to induce immune responses. Baker et al. (35), however, noticed that mice immunized with type III pneumococcal polysaccharide (SSS-III) and treated with anti-lymphocyte serum (ALS), an agent which selectively depletes T cells, resulted in markedly enhanced antibody responses to this antigen. The ALS-induced enhancement of the SSS-III response was abrogated by the infusion of syngeneic thymocytes or further enhanced by the infusion of peripheral blood lymphocytes (22). Based on extensive studies (28) Baker has proposed that two functionally distinct types of thymus-derived cells, suppressor and amplifier cells, act in an opposing manner to regulate the magnitude of the antibody response to SSS-III by B cells.

While assessing the effect of ALS treatment on the humoral antibody response to a panel of differing antigens, Kerbel and Eidinger

(16) showed that ALS treatment of mice immunized with polyvinylpyrrolidone (PVP) resulted in enhanced antibody responses to PVP, a T-independent antigen (11,16,17). Depletion of T cells by adult thymectomy also caused increased serum antibody responses (16,36) and increased numbers of PVP-specific antibody producing cells (37). Thymus implantation or injection of thymus cells partially reversed this effect of adult thymectomy (37). In other studies mice undergoing a mild graft-versus-host reaction had reduced numbers of T cells in their spleens and depressed immune responses to sheep erythrocytes but increased responses to PVP (38). Rotter and Trainin (37) also showed that the magnitude of the PVP-specific antibody response is inversely proportional to the age of the mice studied, i.e., as mice age thymus function declines and the immune response to PVP increases. Collectively, these results suggested that a T cell population exerts a negative regulatory function on the immune response to PVP.

PVP is a useful antigen to use in studies concerning the regulation of immune responses for several reasons: (a) PVP is highly immunogenic in mice and the immune response can be quantified using an antigen binding test (11), a passive hemagglutination assay (11), and more recently, an assay to detect individual cells secreting PVP-specific antibody (37). (b) PVP induces vigorous immune responses in the absence of functional thymus-derived cells but these cells when present appear to regulate the magnitude of the antibody response.

Because PVP does not require the collaboration of helper T cells to induce immune responses, it is relatively easy to demonstrate the effects of other T cell subpopulations on the immune response to this antigen. (c) PVP, a synthetic polymer with many medical and industrial uses (39), is readily available in a variety of molecular weight ranges. Furthermore, PVP is not toxic over a large range of doses (39).

The use of congenitally thymus-deficient nude mice and ALS treatment in conjunction with PVP seemed to offer a useful model system to study regulation of helper T cell independent immune responses. The studies to be presented were designed to develop a sensitive and reproducible method to detect single cells producing PVP-specific antibody, characterize the immune response to PVP in nude and normal mice, and investigate the ability of thymus-derived cells to regulate the immune response to this antigen. Hopefully, the results to be presented here will provide information which can be added to an as yet incomplete picture of immunoregulatory mechanisms.

MATERIALS AND METHODS

Animals

BALB/c mice of both sexes raised in our laboratory or purchased from Texas Inbred Mice Company (Houston, Texas) or Charles River Breeding Laboratory (Wilmington, Massachusetts) were used for most experiments. Congenitally thymus-deficient (nu/nu) mice, hereafter designated nude, were produced from heterozygous stock taken from a line being made congenic with BALB/c mice by cross-intercross mating. The nude mice used in these studies were derived from heterozygous parents of the 11^{th} and 12^{th} cross-intercross generation except for some nudes listed in Table 6 which were either generation 2-4 on the BALB/c background or generation 1-2 on the CFW background.

All mice were 6-12 weeks old at the start of experiments and groups of mice were age and sex matched within a particular experiment. In the case of long-term experiments, the control groups were always carefully age matched.

Antigens and Immunizations

The polyvinylpyrrolidone (PVP) used for immunization (donated by GAF Corporation, New York, N.Y.) had an average molecular weight of 360,000 (designated PVP K90 by the manufacturer). PVP K90 was dissolved in PBS at a concentration of 100 µg/ml and 0.25 ml was injected intravenously via the lateral tail vein resulting in a dose of 0.25 µg/mouse. In some experiments mice were given a prior, weakly

immunogenic priming dose of PVP (0.025 μg) followed 3 days later by an optimally immunogenic challenge dose of PVP (0.25 μg).

Type III pneumococcal polysaccharide was kindly donated by Dr. Phillip J. Baker, Laboratory of Microbial Immunity, National Institutes of Allergy and Infectious Diseases, NIH, Bethesda, Maryland. The immunologic properties of the SSS-III used and the method by which it was prepared have been described (40-43). Mice were primed with 0.01 μg of SSS-III i.v. and subsequently challenged with a mixture of 1.0 μg of SSS-III and 0.25 μg of PVP in saline.

Burro erythrocytes (BE) were obtained from Colorado Serum Company, Denver. Mice were given a single i.v. injection of 0.2 ml of a 10% suspension of washed BE in PBS.

The Vi antigen (kindly donated by Dr. F. G. Jarvis, Idaho State University) used in these studies was purified form Citrobacter freundii by an electrophoretic procedure. Mice were injected i.v. with 1 μg Vi in 0.2 ml of saline; the number of Vi-specific PFC was determined 4 days following immunization.

Lipopolysaccharide (LPS) was extracted from E. coli and was supplied by Dr. J. A. Rudbach, University of Montana, Missoula, Montana. Mice were given 1 μg LPS in saline i.v. and the magnitude of the LPS-specific PFC response was determined 5 days following immunization.

N-vinyl-2-pyrrolidone and 2-Pyrrolidone

N-vinyl-2-pyrrolidone (NVP) and 2-pyrrolidone (2-PY) were purchased from K & K Laboratories, Plainview, New York. NVP and 2-PY were used in plaque inhibition experiments in which varying amounts of one or the other were incorporated in the agar layer together with PVP immune spleen cells and PVP-coated SE.

Concanavalin A, Hydrocortisone Acetate, and Thymosin

Concanavalin A (Con A) was purchased from Miles Laboratories, Elkhart, Indiana, or in the case of the experiment presented in Table 21, Con A was kindly donated by Dr. Phillip J. Baker Laboratory of Microbial Immunity, NIAID, NIH, Bethesda, MD.

Hydrocortisone acetate (HC) suspension was purchased from Wollins Pharmacal Corp., Farmingdale, NY. HC was given as a single i.p. injection consisting of 2.5 mg/mouse.

Thymosin fraction 5 was produced by Hoffman-LaRoche, Inc., Nutley, NJ for Dr. A. L. Goldstein, University of Texas and kindly donated by Dr. J. T. Ulrich, Department of Human Biological Chemistry and Genetics, Division of Biochemistry, The University of Texas Medical Branch, Galveston, TX. Approximately 73 mg of salt-free lyophilized thymosin, reported to be endotoxin free, was reconstituted with 5 ml of sterile normal saline. The restored thymosin was then divided into 9 equal aliquots and frozen at -60 C. Immediately prior to use,

an aliquot was thawed and 3.7 ml of cold PBS added. Mice were injected i.p. with 0.25 ml of the diluted thymosin to give about 500 µg of thymosin per mouse. Injections were given on Monday, Wednesday, and Friday for 3 weeks prior to immunization.

Antithymocyte and Antilymphocyte Serum

Rabbit antimouse thymocyte serum (ATS) and rabbit antimouse lymphocyte serum (ALS) were purchased from Microbiological Associates, Bethesda, MD, or ATS was prepared by injecting 5×10^8 BALB/c thymocytes i.v. into each of 3 rabbits at weekly intervals for 3 weeks. Seven days following the last injection the rabbits were bled and their serum harvested, pooled, and frozen in 3 ml portions at -20 C. ATS, ALS, or normal rabbit serum (NRS) was given as a single i.v. injection at the time of immunization of mice; the amount and lots of ATS, ALS, or NRS used are given at appropriate places in the text.

Plaque Assays

PFC specific for PVP, SSS-III, or BE were detected by a slide modification (44) of the localized hemolysis in gel technique. BE, or sheep erythrocytes coated with PVP (17,37) or SSS-III (45) were used as indicator cells. Routinely, the magnitude of the PFC response to each antigen was determined 5 days following challenge immunization.

The procedure for coating SE with PVP for use in plaque assays was adapted from a technique reported by Rotter and Trainin (37). SE

are washed 3 times in PBS to yield 2 ml of packed cells and resuspended to a 5% suspension by adding 38 ml of PBS to the packed cells. Forty ml of 0.1 mg/ml tannic acid (TA) in PBS is added to the 40 ml of 5% SE and incubated at room temperature for 15 min. TA is prepared by dissolving 100 mg TA in 1 liter of PBS and allowing the solution to remain at room temperature for 10 hr. The aged TA is divided into 40 ml portions and stored at -20 C until just prior to use.

The tannic acid treated SE (T-SE) are separated into 2 equal portions in 50 ml plastic centrifuge cups and washed 3 times by centrifugation in PBS at room temperature. One portion, consisting of 1.0 ml of packed T-SE is resuspended to a 5% suspension in PBS and the other 1.0 ml of packed T-SE is resuspended to a 1:11 suspension in PBS for use as the SE control in the plaque assay. Twenty ml of 0.1 mg/ml PVP (K&K Laboratories, pharmaceutical grade) is added to the 20 ml of 5% T-SE. The suspension is mixed and incubated at room temperature for 15 min. Finally, the PVP-coated SE (PVP-SE) are washed 3 times in PBS and resuspended to a 1:11 suspension in PBS for use as the indicator cells to detect PVP-specific PFC.

PVP Passive Hemagglutination Assay

A microtiter assay was used to determine the titer of PVP-specific serum antibody. SE are coated with PVP as described above but resuspended in PBS containing 0.4% gelatin to a 0.25% suspension.

Two fold serial dilutions of serum are made in 0.025 ml of PBS containing 0.4% gelatin. The gelatin stabilizes the PVP-coated SE (46) allowing the cells to settle into recognizable patterns.

Irradiation of Mice

Mice were irradiated using a ^{60}Co source in a slowly revolving plastic holder which immobilized mice in individual compartments. The skin source distance was always 60 cm; the dose rate and final dosage given are listed in appropriate places in the text.

Cell Transfers

Single cell suspensions were prepared from the spleen, thymus, or bone marrow of PVP-primed or normal mice. The cells were washed once in balanced salt solution, resuspended to the appropriate concentration, and injected i.v. in a total volume of 0.5 ml. Recipients were normal or nude mice or normal mice which had been lethally irradiated about 16 hours previously. In some cases, mice were given a separate i.v. injection of PVP or BE at the same time as cell transfer.

Adult Thymectomy

Mice were thymectomized at 5-6 weeks of age according to a method described by Morse et al. (47). A 1-2 cm ventral midline incision was made through the skin. The submaxillary gland was teased up and a 1 cm incision was made through the sternum with scissors.

The ribs were spread using small self retaining retractors (George Tiemann and Co., NY) and the thymus was removed by suction through a pasteur pipette with an enlarged opening. The wound was closed using 2 or 3 auto clips in the skin. Sham thymectomized mice received exactly the same treatment without removal of the thymus. Each animal was examined macroscopically for the presence of thymic remnants when sacrificed for PFC assay.

Thymus Gland Grafting

Neonatal BALB/c thymus glands were implanted under the renal capsule of nude or normal mice by previously described techniques (48-50). In most cases one thymus gland was placed under each kidney capsule of recipients. Mice were used in experiments 8 weeks following grafting and each mouse was checked for the presence of a thymus gland under the capsule at the time of plaque assay.

Presentation of Data and Statistical Methods

In most cases results are presented as the arithmetic mean PFC/spleen ± standard deviation (SD) derived from mice within a treatment group. However, in some experiments the results are presented as Log_{10} PFC/spleen ± SD in an effort to normalize the variance since with PFC values the standard deviation is proportional and varies directly as the mean (51). The geometric mean is given in parenthesis below the Log_{10} value for each group to facilitate observation of the

data. Student's t test was used to assess the significance of the differences noted in the magnitudes of the PFC responses in those experiments in which the responses of different groups of mice were compared. Differences between mean values for groups of mice were considered to be significant when probability (p) values less than 0.05 were obtained.

RESULTS

Treatment of SE with PVP and TA for Use in the PFC Assay

Initial experiments were performed to determine the optimal conditions to coat SE with PVP to detect PVP-specific PFC. Figure 1 shows a diagrammatic representation of the structure of PVP and Table 1 lists the designations and molecular weights of those preparations available for use. Preliminary experiments and a previous report (37) indicated that mice produce appreciable numbers of PFC 5 days following immunization with 0.25 μg of PVP K 90 and that PVP K 30 would successfully coat SE for use in the plaque assay. To determine the optimal concentration of K 30, SE were treated with TA and then divided into 8 aliquots. An equal volume of an appropriate concentration of K 30 was mixed with each portion of SE. The cells were incubated, washed, and plated with PVP-immune spleen cells. The results (Table 2) show that the greatest number of plaques were observed using SE coated with 0.1 mg/ml of PVP K 30. The quality of the plaques was also optimal using this concentration.

Successful coating of SE with PVP depends on prior treatment of SE with a dilute solution of TA. Fresh solutions of TA are colorless gradually turning an increasingly green color upon aging. It was possible, therefore, that the age of the TA used to treat SE could affect the quality or the numbers of PVP-specific PFC detected. To test this possibility, washed SE were divided into 8 equal portions and treated

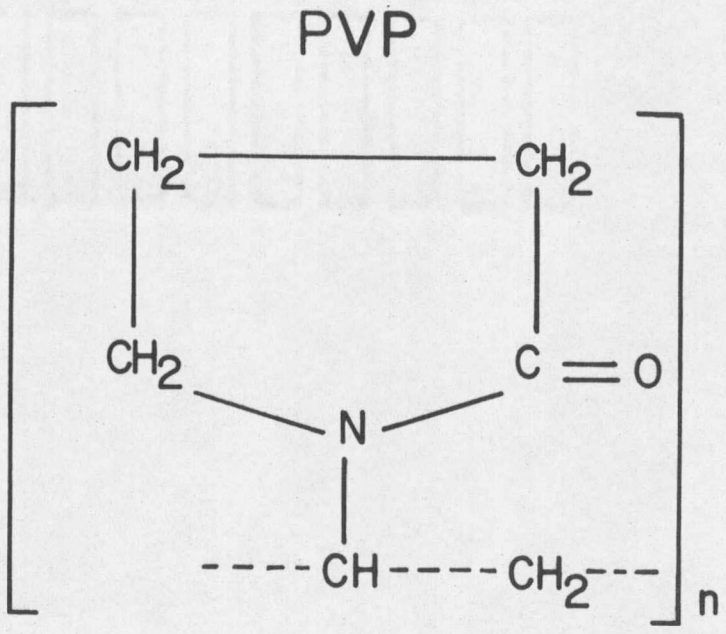

Figure 1. Diagrammatic Representation of Polyvinylpyrrolidone.

Table 1. Designation and Average Molecular Weights of PVP Preparations Presently Available

PVP Designation	Average Molecular Weight	2-Pyrrolidone Monomer Units/Molecule
PVP K 90	360,000	3,240
PVP K 60	160,000	1,440
PVP K 30	40,000	360
PVP K 15	10,000	90

Table 2. The Effect of Varying the Concentration of PVP Used to Coat SE on the PVP-Specific Plaques Observed[a]

PVP Concentration mg/ml	PVP-Specific PFC			
	Direct		Facilitated[b]	
	Spleen	10^6	Spleen	10^6
0	200	1	250	1
0.002	12,750	58	16,000	73
0.01	33,750	153	35,500	161
0.02	36,250	165	35,000	159
0.1	38,500	175	39,500	180
0.2	22,750	103	25,750	117
1.0	25,750	117	26,000	118
2.0	23,500	107	26,000	118
20.0	23,000	105	23,000	105

[a]Three BALB/c mice, 4 months old, were injected with 0.25 μg of PVP and 5 days later their spleen cells were pooled and the number of direct and facilitated PVP-specific PFC was determined using SE coated with varying concentrations of PVP K30.

[b]The number of facilitated PFC/spleen was determined by treating duplicate slides with a solution of complement and rabbit antimouse γ-globulin serum.

with TA preparations of varying ages. Upon incubation the cells were washed and the separate portions incubated with 0.1 mg/ml PVP K 30. The cells were again incubated, washed, and plated with PVP-immune spleen cells. The results presented in Table 3 clearly show that the age of the TA used to treat SE does affect both the quantity and the quality of the PVP-specific plaques detected. Twelve hours was chosen as the optimal time to age TA. SE treated with TA aged for 12 hours showed numbers of plaques close to the maximum detected, but more importantly, the plaques were sharp and easy to distinguish.

Specificity of PVP-Induced Plaques

To determine if PFC observed following PVP immunization were producing antibody that would specifically bind PVP, plaque inhibition studies were performed. Accordingly, groups of mice were given an optimally immunogenic dose of either PVP, Vi antigen (Vi), or lipopolysaccharide (LPS). Five days later the mice were killed and PVP immune spleen cells were plated with PVP-coated SE in the presence of varying amounts of free PVP, Vi immune spleen cells were plated with Vi-coated SE in the presence of varying amounts of free PVP, and LPS immune spleen cells were plated with LPS-coated SE in the presence of varying amounts of free PVP. The results (Figure 2) show that PVP plaques are markedly inhibited by the addition of free PVP to the overlay whereas the number of Vi and LPS plaques was not affected by the addition of

Table 3. The Effect of Varying the Age of the Tannic Acid Solution Used to Treat SE on PVP-Specific Plaques Observed

Age of Tannic Acid (Hours)	PVP-Specific PFC/Spleen[a]	Plaque Quality[b] (Scale 1-5)
0	31,500	2
4	35,438	3
8	34,750	3
12	32,688	4
20	32,188	4
30	13,500	1
50	313	--
PBS	125	--

[a]Three BALB/c mice, 4 months old, were injected with 0.25 µg of PVP and 5 days later their spleen cells were pooled and the number of direct PVP-specific PFC determined using SE treated with tannic acid which had been aged for varying time periods. Following tannic acid treatment all SE portions were treated with PVP K30 in an identical manner.

[b]The quality of plaques observed was judged on an arbitrary scale of 1-5; the poorest plaques were assigned scores of one and the better plaques assigned increasingly higher values.

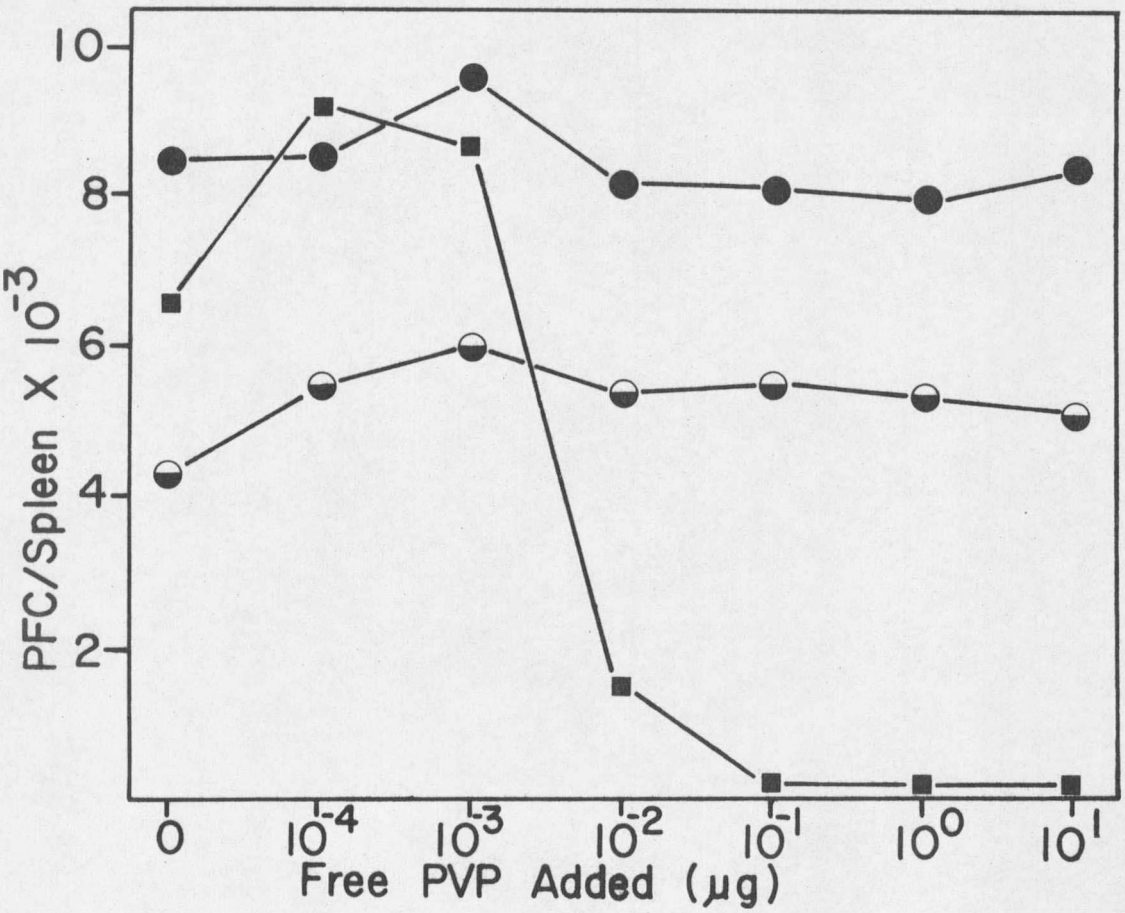

Figure 2. Specificity of PFC Detected Using PVP-Coated SE. Pooled spleen cells from groups of mice immunized with PVP (■—■), Vi (◓—◓), or LPS (●—●) were plaqued with the appropriate antigen-coated SE in the presence of varying concentrations of PVP K90.

PVP. The inhibition of only PVP-specific plaques indicates that PVP did not inhibit plaque formation nonspecifically. In those slides showing plaque inhibition, PVP-specific antibody released from potential PFC bound the free PVP in the overlay and was prevented from reaching and subsequently, upon the addition of complement, lysing the PVP-coated SE.

Using the plaque inhibition assay it was also possible to partially investigate the nature of the antigenic determinant or epitope of the PVP molecule. PVP immune spleen cells were plated with PVP-coated SE in the presence of varying amounts of either 2-pyrrolidone (2-PY), N-vinyl-2-pyrrolidone (NVP), or PVP K 90. The results (Figure 3) demonstrate that 2-PY, which is the basic heterocyclic pyrrolidone ring structure, fails to inhibit PVP-specific plaques at any concentration tested. However, NVP, a monomer which consists of the pyrrolidone ring and a single vinyl group, inhibits PVP-specific plaques almost as efficiently as PVP K 90, the original antigen used to stimulate the immune response. These results indicate that the vinyl group is an integral part of the PVP epitope.

The plaque inhibition procedure can also be used to determine the avidity of antibodies produced by single hemolytic plaque-forming cells (52,53). The avidity of the antibodies produced by the PFCs is reflected by the relative sensitivity of the plaques to inhibition by free antigen. Plaques formed by low avidity antibody require

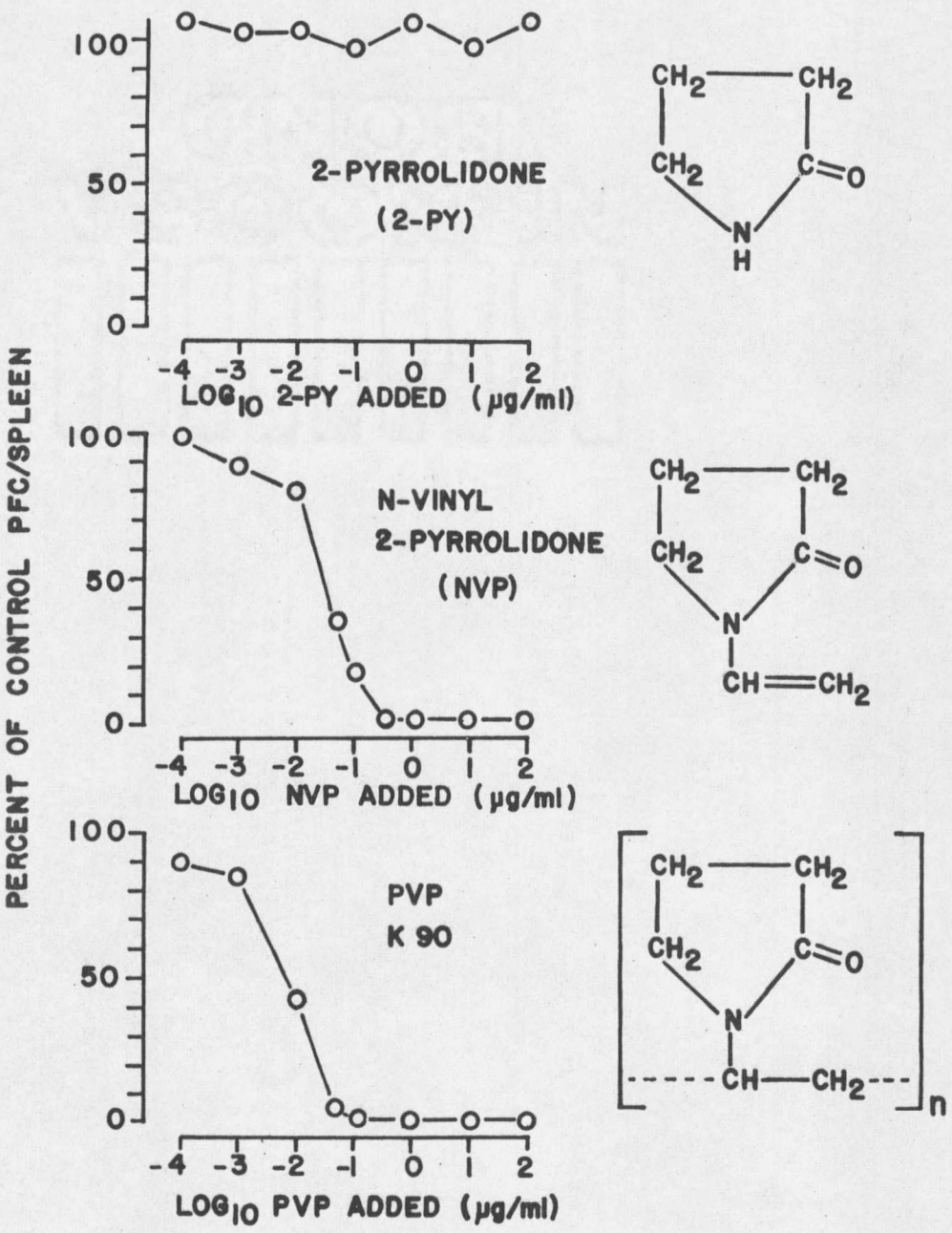

Figure 3. Inhibition of PVP-Specific Plaques with PVP K90, N-Vinyl-2-Pyrrolidone, and 2-Pyrrolidone. A pool of spleen cells derived from 3 BALB/c mice given 0.25 µg of PVP 5 days previously was plaqued in the presence of varying amounts of 2-PY, top; NVP, middle; or PVP, bottom.

higher antigen concentrations for inhibition than high avidity plaques. PVP appears to stimulate relatively high avidity plaques compared to plaques induced to the protein, bovine serum albumin (BSA). Approximately 0.01 μg/ml PVP inhibited 50% of PVP plaques (Figure 3) whereas Andersson (52) has reported that about 10 μg/ml of BSA must be added to inhibit 50% of BSA-specific PFC.

Class of Antibody Produced by PVP-Specific PFC 5 Days Following Immunization

Preliminary experiments demonstrated that treatment of slides with a rabbit antimouse γ-globulin serum did not result in increased numbers of PVP-specific PFC detected. The preparation and dilution of facilitating serum used had been previously shown to be effective in visualizing indirect PFC to sheep erythrocytes. To further define the class of antibody produced by PFC-specific PFC 5 days following immunization, normal mice were given a single optimally immunogenic dose of PVP; also a group of normal mice was given a primary and secondary immunization of SE. Five days following the last immunization, all mice were sacrificed and their spleen cells plaqued on duplicate slides. One set of slides was treated with Con A (54) before the addition of complement or complemtn plus facilitating serum.

The data of Table 4 show that treatment with Con A of slides from normal mice immunized twice with SE resulted in a dramatic reduction of direct plaques but only partial reduction of facilitated plaques. The

Table 4. Inhibition of IgM-Producing PFC by Concanavalin A

Antigen	Inhibitor[b]	PFC/Spleen[a] Direct	Facilitated
SE	None	37,000	158,750
	Con A	150	103,750
PVP	None	5,000	ND[c]
	Con A	0	ND

[a]Direct or facilitated PFC/spleen of mice immunized 5 days previously with 0.25 µg of PVP or 2 injections of 0.2 ml of a 10% suspension of SE spaced 5 days apart. Pooled spleen cell suspensions from 3 mice were used for all determinations.

[b]Spleen cell suspensions were plaqued in the normal way except that slides were treated with Con A before the addition of complement. Control slides which received no inhibitor were treated with saline before the addition of complement.

[c]Not determined.

reduction in the number of plaques detected following inhibitor treatment most probably was due to the inhibition of plaques around IgM-producing cells. When immune spleen cells from PVP-immunized mice were treated with Con A, no PVP-specific plaques were detected. These results confirm that the direct PVP-specific PFC detected 5 days following immunization are the result of IgM-producing cells. Since no increase in PFC has ever been observed using facilitating serum, it was not necessary to test the effect of Con A treatment of slides treated with an antimouse γ-globulin serum. These results, however, apply only to PFC detected 5 days following immunization; it is possible that cells producing antibody of classes other than IgM arise later in the course of the antibody response to PVP.

Passive Hemagglutination Assay for PVP-Specific Serum Antibody

To measure PVP-specific serum antibody, a passive hemagglutination assay using microtiter plates and diluting loops was developed. Initial attempts to demonstrate agglutination of PVP-coated cells failed because these cells did not settle into recognizable patterns (Table 5). When PVP-coated SE were resuspended in PBS containing 0.4% gelatin (46), the cells formed easily distinguishable agglutination patterns. Varying concentrations of PVP-coated SE in gelatin were added to identical serum sample titrations to determine the concentration of PVP-coated SE which would yield the maximum titer and

Table 5. Utilization of PVP-Coated SE in a Passive Hemagglutination Assay

PVP-SE Resuspended in[a]	Concentration of PVP-SE[b]	PHA Titer[c]	Comment
PBS	1.0 %	--	Not Readable
	0.5 %	--	Not Readable
	0.25 %	--	Not Readable
	0.125 %	--	Not Readable
	0.06 %	--	Not Readable
PBS + 0.4 % Gelatin	1.0 %	8	Easily Readable
	0.5 %	16	Easily Readable
	0.25 %	64	Easily Readable
	0.125 %	64	Difficult to Read
	0.06 %	64	Difficult to Read

[a] SE were coated with PVP in the normal manner and resuspended to varying concentrations in either PBS or PBS + 0.4 % gelatin.

[b] 0.025 ml of varying concentrations of PVP-coated SE were added to the wells in each row.

[c] Reciprocal of the highest dilution of serum showing a clearly recognizable agglutination pattern. Identical 2-fold serial dilutions in PBS or PBS + 0.4 % gelatin were made in microtiter plates of a pool of serum derived from mice immunized with 0.25 µg of PVP 5 days previously.

yet be easily readable and reproducible. Table 5 shows that 0.25% of PVP-coated SE in 0.4% gelatin was the best concentration tested. More concentrated suspensions were not as sensitive to small amounts of antibody and more dilute suspensions were sensitive but difficult to read due to the very small number of cells present.

PVP Dose Response

Before meaningful experiments using PVP could be performed, various characteristics of the PVP-specific immune response had to be determined. To establish the dose response characteristics of the primary PFC response to PVP, mice were injected with PVP doses ranging from 10^{-4} µg to 10^{2} µg; the number of PVP-specific PFC was determined 5 days later. The results (Figure 4) show that mice produce appreciable numbers of PFC in response to only a narrow range of PVP doses (10^{-1} to 10^{0} µg). Subsequent experiments indicated that the greatest number of PFC was produced in response to 0.25 µg of PVP; this dose was considered to be the optimal dose. It can be seen from the dose response curve (Figure 4) that a PVP dose of 10^{-2} µg and 10^{-4} µg were not consistently detectable. Immunization with doses greater than 1.0 µg resulted in a reduction in the magnitude of the PFC response which may be attributed to the induction of high dose paralysis (55).

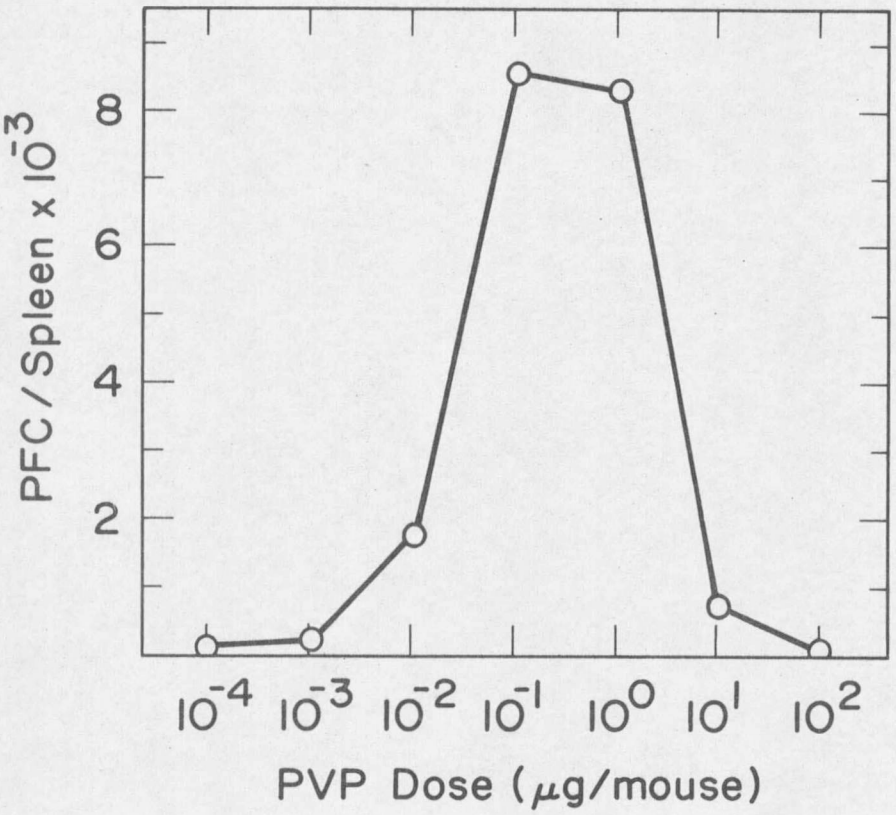

Figure 4. PVP-Specific PFC Response Determined 5 Days Following Immunization of Mice with Varying Doses of PVP. Each point represents the mean PFC/spleen of 8 similarly treated mice.

PVP-Specific PFC Response on Varying Days Following Immunization

Groups of mice were injected with an optimally immunogenic dose of PVP on varying days before plaque assay and the number of PFC determined on the same day for all mice. The results presented in Figure 5 show that mice produce very few PVP-specific PFC 2 and 3 days following immunization and produce maximal numbers of splenic PFC 4 days after immunization. The magnitude of the PFC response 5 days after immunization was found to be similar to the peak response observed on day 4 post immunization. Because of the very drastic reduction in plaques observed just prior to day 4 and the maintenance of peak PFC responses on days 4 and 5, the number of PVP-specific PFC was routinely determined approximately 4-1/2 days following immunization. The number of PFC declined on days 6 and 7 following immunization.

PVP-Specific Serum Antibody Response on Varying Days Following Immunization

The serum antibody response to a single, optimally immunogenic dose of PVP was measured on varying days following immunization; similar results were obtained using a microtiter and a tube agglutination procedure. Tube agglutination results (Figure 6) show that a maximal PVP-specific serum antibody titer is present 5 days following immunization. Thereafter, the titer remains at approximately the same level through day 50 following immunization. To discriminate between IgM and

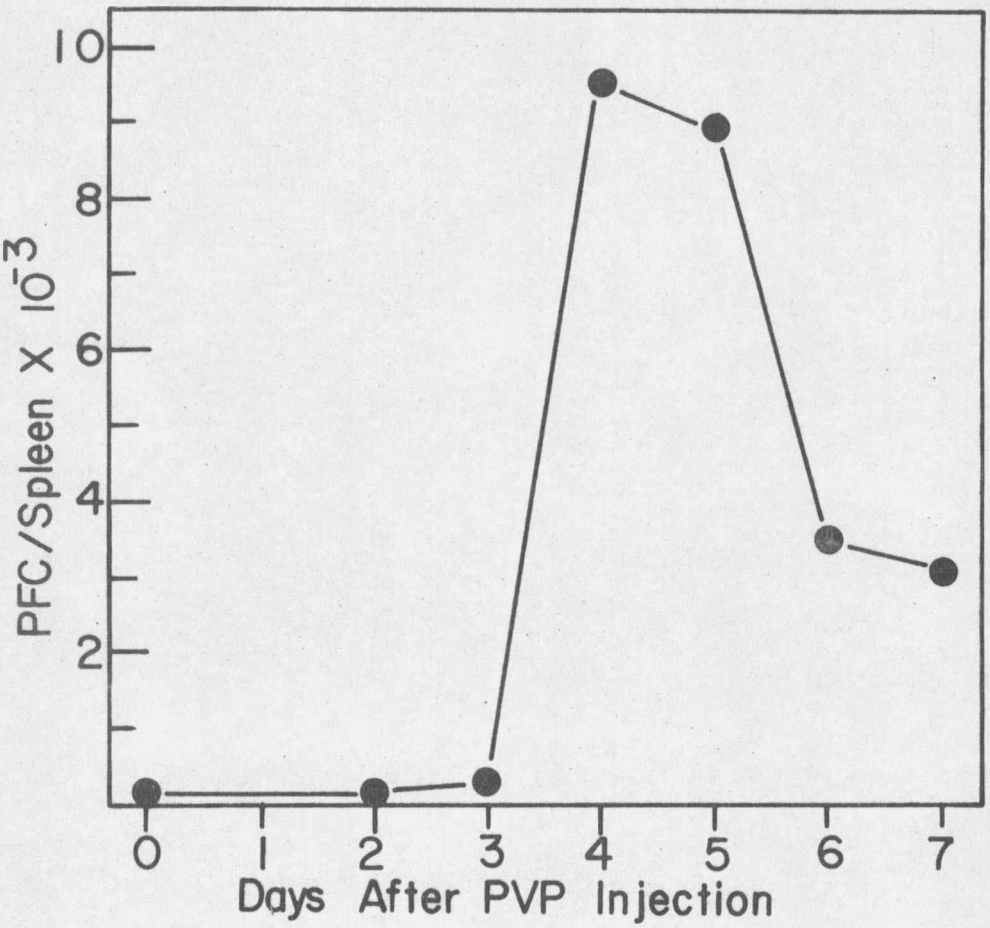

Figure 5. PVP-Specific PFC Response on Varying Days Following Immunization with 0.25 µg of PVP. Each point represents the mean PFC/spleen of 4 mice.

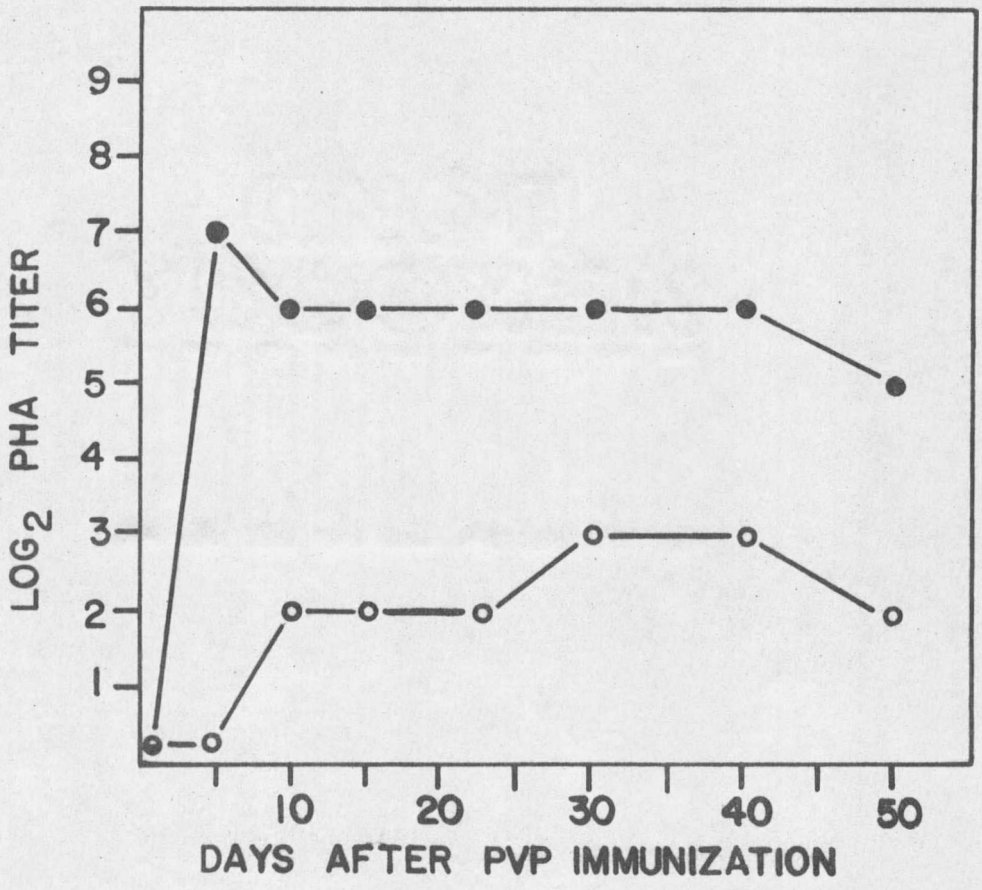

Figure 6. PVP-Specific Serum Antibody Response on Varying Days Following Immunization. Ten BALB/c mice were immunized with 0.25 μg of PVP and bled on varying days following immunization. Serum from each bleeding was pooled and samples were titered without further treatment (●—●) or samples were incubated with 0.1 M 2-ME for 1 hour at room temperature just prior to titration (O—O).

IgG serum antibody, 0.1 M 2-mercaptoethanol (2-ME) was incubated with serum samples for 1 hour at room temperature (56,57). The 2-ME treated serum was then titered in the normal manner. Agglutination following 2-ME treatment has been attributed to IgG antibody. On day 5 following PVP immunization, no 2-ME resistant antibody was detected, but on all other days tested, there appeared to be a small amount of 2-ME resistant antibody present.

PVP-Specific PFC Response of Nude and Normal Mice

Because a previous study (11) using artifically T cell-deprived mice indicated that PVP was able to induce vigorous immune responses in the absence of T cells, it was important to evaluate the immune response to PVP in congenitally thymus-deficient (nude) mice. Nude and normal mice were injected with an optimally immunogenic dose of PVP, and the number of spleen cells producing antibody specific for PVP was determined 5 days later. Table 6 presents the results of experiments 1 through 4 using different groups of nude and normal mice. In experiment 1, nude and normal littermate mice were taken from a colony maintained under conventional conditions and consisted of mice derived from heterozygous parents of the second to fourth cross-intercross generation. Experiment 2 represented nude mice being bred into the CFW strain and normal CFW mice. The nude mice used in experiments 3 and 4 were derived from the mating of heterozygous females

Table 6. PVP-Specific PFC Response in Normal and Nude Mice Given an Optimally Immunogenic Dose of PVP

Exp. No.	Mice	No. of Mice	PVP-Specific PFC/Spleen ± SD[a]	P
1	NLM (BALB/c)	15	3.666 ± 0.112[b] (4,634)[c]	$p > 0.2$[d]
	Nude (BALB/c)	16	3.577 ± 0.067 (3.766)	
2	Normal (CFW)	9	3.841 ± 0.09 (6,934)	$p > 0.1$
	Nude (CFW)	21	3.616 ± 0.08 (4,130)	
3	Normal (BALB/c)	16	4.183 ± 0.05 (15,240)	$p < 0.005$
	Nude (BALB/c)	15	3.637 ± 0.15 (4,335)	
4	Normal (BALB/c)	11	4.133 ± 0.06 (13,583)	$p > 0.05$
	Nude (BALB/c)	11	3.932 ± 0.07 (8,550)	

[a] Number of PVP-specific PFC/spleen detected 5 days after immunization with 0.25 μg of PVP.

[b] Log_{10} ± SD for 9-21 similarly treated mice.

[c] Geometric mean.

[d] Probability value comparing the mean PFC/spleen of normal and nude mice using Student's t test.

and thymus-gland grafted nude males of the 11^{th} and 12^{th} cross-intercross generations. The nude mice in experiment 3, however, were taken from a colony maintained in a relatively dirty environment whereas the nude mice of experiment 4 were raised in a clean environment in a separate building from the main mouse rooms. Although the genetic background of these mice was the same, the nude mice used in experiment 4 were generally much healthier, weighed more, and lived longer than the nude mice from the colony used in experiment 3.

The results of experiment 1 and 2 show that nude mice responded to PVP in a manner quantitatively similar to thymus-bearing littermates or to normal CFW mice; the differences between the two groups were not significant in either experiment 1 or 2 ($p > 0.2$ and $p > 0.1$, respectively).

In experiment 3, nude mice had significantly lower numbers of PVP-specific PFC when compared to normal mice but in experiment 4, using healthy nude mice the magnitude of the response of nude mice was statistically the same as the response of normal mice. These data suggest that functional thymus-derived cells are not an absolute requirement in the immune response of mice to PVP. The apparent difference noted in experiment 3 could have been due to the poor health of the nude mice used. However, the condition of mice used in these experiments was not monitored in any quantitative manner but rather, mice were arbitrarily judged according to external appearance and

vigour. Thus, other possibilities which will be discussed below do exist and cannot be ruled out by the results presented here.

The Effect of Varying Doses of Radiation on the Immune Response to PVP

Although thymus-derived cells are not an absolute requirement in the immune response to PVP, previous reports (16,36-38,58) suggested that these cells can regulate the magnitude of the immune response to this antigen. To test this possibility we attempted to eliminate T cells that would normally exert a negative influence on the PVP response (suppressor T cells) by treating mice with varying doses of radiation. This approach was shown to increase IgE responses of rats to DNP-ascaris presumably by eliminating suppressor T cells (59).

Accordingly, groups of mice were given varying doses of radiation and immunized with PVP. The number of PVP-specific PFC was determined 5 days later. The results (Table 7) show that even a dose as low as 200 rad resulted in a dramatic reduction of the immune response to PVP and doses of 400, 600, and 800 rad totally abrogated PVP-specific PFC responses 5 days following radiation and immunization. These results seem to indicate that low doses of radiation kill B cells reactive to PVP before T cells that might exert regulatory influences on the immune response to this antigen. However, it is possible that lower doses of radiation or other treatment protocols would have resulted in more meaningful results.

Table 7. The Effect of Varying Doses of Radiation on the PVP-Specific PFC Response in BALB/c Mice

Group	No. of Mice	Treatment[a]	PVP-Specific PFC/Spleen
A	3	None	17,950
B	3	200 rad	2,075
C	3	400 rad	8
D	3	600 rad	0
E	3	800 rad	0

[a]Mice were given varying doses of radiation from a ^{60}Co source. Within 3 hours after irradiation all mice were immunized with 0.25 µg of PVP. The number of PVP-specific PFC was determined 5 days later.

Effect of ATS or ALS Treatment on the PFC Response to PVP

Treatment of mice with ATS or ALS, agents which cause a depletion of thymus-derived cells, causes a depression of thymus-dependent immune responses but not thymus-independent immune responses (60). Thus, if suppressor T cells normally function to dampen the response to PVP, their depletion following ATS or ALS treatment should result in increased PVP-specific PFC responses.

To test this possibility groups of normal mice were given a single i.p. injection of ATS or ALS at the time of i.v. immunization with PVP. The number of PVP-specific PFC was determined 5 days later. The results (Table 8) demonstrate that using this treatment protocol and either ATS or ALS with different strains of mice, no significant enhancement of the PVP-specific PFC response was observed.

It was possible that to be effective ATS must be administered by the same route as antigen. Accordingly, BALB/c mice were injected i.v. with an optimally immunogenic dose of PVP; immediately after immunization, various amounts of ATS or ALS were administered i.v. to some of the mice. The PFC response to PVP was assessed 5 days later. The data (Table 9) show that the magnitude of the PFC response to PVP was markedly increased in all groups of mice given any dose of ATS or ALS. The ATS or ALS-induced increase in the PFC response is apparent when the data are expressed as PFC/spleen or as PFC/10^6 spleen cells.

Table 8. The Effect of Intraperitoneal Injection of ALS or ATS on the PVP-Specific PFC Response[a]

Experiment	Strain	Treatment	PVP-Specific PFC/Spleen[b]
1	LM	--	4,500
	LM	ATS	9,238
2	LM	--	4,800
	LM	ALS	4,556
3	BALB/c	NRS	1,530
	BALB/c	ALS	2,390
4	CFW	--	3,313
	CFW	ATS	3,413

[a]Mice were given a single i.p. injection of ATS (Microbiological Associates, Lot No. 14580) or 0.3 ml of ALS (Microbiological Associates, Lot No. 14031) at the time of i.v. immunization with 0.25 µg of PVP. In some cases control mice were given 0.3 of normal rabbit serum (NRS). The number of PVP-specific PFC was determined 5 days following immunization.

[b]Mean PFC/spleen for 4-6 similarly treated mice.

Table 9. Effect of Treatment with ATS or ALS on the Magnitude of the PFC Response to PVP in Normal BALB/c Mice

Exp. No.	Treatment of Mice	PVP-Specific PFC[a]	
		Spleen	10^6
I	None	3.845 ± 0.183[b]	1.790 ± 0.105
		(6,998)[c]	(62)
	ATS, 0.10 ml	4.630 ± 0.329	2.340 ± 0.257
		(42,658)	(219)
	ATS, 0.20 ml	4.847 ± 0.114	2.635 ± 0.139
		(70,307)	(432)
	ATS, 0.30 ml	4.759 ± 0.306	2.423 ± 0.249
		(57,412)	(265)
	ATS, 0.40 ml	4.861 ± 0.168	2.537 ± 0.163
		(72,611)	(344)
II	None	3.931 ± 0.125	1.898 ± 0.120
		(8,531)	(79)
	ALS, 0.25 ml	4.487 ± 0.271	2.404 ± 0.168
		(30,690)	(254)
	ALS, 0.50 ml	4.590 ± 0.531	2.396 ± 0.410
		(38,904)	(249)

[a] Numbers of PVP-specific PFC per spleen and per 10^6 spleen cells detected 5 days after immunization with 0.25 μg PVP and i.v. injection of varying amounts of ATS or ALS. ATS, Microbiological Assoc., Lot 14580; ALS, Microbiological Assoc., Lot 14031.

[b] Log_{10} ± standard deviation for 3-4 similarly treated mice.

[c] Geometric mean.

Therefore, the increase is not merely the result of ALS-induced splenomegaly.

ALS Treatment of Mice Immunized with both PVP and SSS-III

Baker (28,35) has demonstrated that the magnitude of the PFC response to SSS-III is greatly enhanced in mice treated with ALS. The experiments reported here show that the magnitude of PFC responses to PVP are also markedly elevated in mice treated with ATS or ALS compared to untreated control mice. It was interesting to evaluate the effect of ALS treatment on the magnitude of the PFC response to both PVP and SSS-III in mice given an optimally immunogenic dose of both antigens. To perform this experiment, BALB/c mice were given a mixture of 0.5 μg of PVP and 0.5 μg of SSS-III by a single i.p. injection. Half of the mice were then injected i.p. with a preparation of horse anti-mouse lymphocyte serum kindly donated by Dr. Phillip Baker, Laboratory of Microbial Immunity, NIAID, NIH. The number of PFC to each antigen was detected 5 days following immunization and ALS treatment.

The results (Table 10) show that ALS treatment of doubly immunized mice induced an 18-fold enhancement in the magnitude of the PFC response to PVP and a 14-fold enhancement in the magnitude of the PFC response to SSS-III when compared to mice which were given only PVP and SSS-III. These results showing that the magnitude of the PFC response to both antigens is increased to approximately the same degree

Table 10. Effect of ALS Treatment on the Magnitude of the PFC Response to PVP and SSS-III

Group	No. of Mice	Treatment	PFC/Spleen[a]	
			PVP-specific	SSS-III-specific
A	4	PVP + SSS-III	6,444	6,950
B	4	PVP + SSS-III + ALS	118,213	98,088

[a]Numbers of PVP-specific and SSS-III-specific PFC/spleen detected 5 days following i.p. immunization of mice with a mixture of 0.5 μg of PVP and 0.5 μg of SSS-III. Mice in group B were also given an i.p. injection of 0.3ml of ALS (donated by Phillip J. Baker, Laboratory of Microbial Immunity, NIAID, NIH, Bethesda, Maryland).

suggested that ALS-induced enhancement is not antigen specific but rather that depletion of T cells by ALS treatment leads nonspecifically to increased PFC responses to certain T helper cell independent antigens. Thus, this phenomena is not restricted to a single unique antigen. However, ALS-induced enhancement may only be demonstrable with certain thymus-independent antigens which share some as yet unidentified characteristics.

Effect of ALS Treatment on the PFC Response to PVP and BE in Normal Mice

If ALS acts by depletion of thymus-derived cells, it should be possible to demonstrate enhancement of the PFC response to PVP under conditions in which the PFC response to the thymus-dependent antigen, BE, was suppressed. In this experiment, 10 BALB/c mice were each immunized with both PVP and BE and five of these mice were also injected with ALS. The data of Table 11 show that, in doubly immunized mice, treatment with ALS caused a significant increase in the response to PVP ($p<0.001$) and a significant decrease in the response to BE ($p<0.001$).

It is possible that ATS or ALS, while impairing the thymus-dependent response to BE by inactivation of helper T cells, could enhance the response to PVP, in which there is no absolute requirement for thymus-derived cells, by removing a population of suppressor cells

Table 11. Effect of Treatment with ALS on the Magnitude of the PFC Response to PVP and BE in Normal BALB/c Mice

Treatment of Mice	PVP-Specific PFC[a]		BE-Specific PFC[a]	
	Spleen	10^6	Spleen	10^6
None	3.918±0.125[b]	1.791±0.151	4.396±0.210	2.270±0.237
	(8,279)[c]	(62)	(24,889)	(186)
ALS,[d] 0.50 ml	4.669±0.171	2.636±0.206	3.468±0.260	1.429±0.242
	(46,666)	(433)	(2,938)	(27)

[a] Numbers of PVP-specific PFC and BE-specific PFC detected 5 days after immunization of all mice with 0.25 µg PVP and 0.2ml of a 10% suspension of BE.

[b] Log_{10} ± standard deviation for 5 similarly treated mice.

[c] Geometric mean.

[d] ALS, Microbiological Associates Lot 14031.

or by a direct stimulatory effect upon B cells. The following experiment was designed to distinguish between these possibilities.

Effect of ATS on the PFC Response to PVP in Normal and Nude Mice

Nude and normal BALB/c mice were immunized with PVP, injected with either ATS or NRS, and the magnitude of the PFC response to PVP was assessed 5 days later. The normal group receiving ATS had a 7.5 fold greater PFC response than the NRS-treated normal group; in contrast, ATS treatment did not increase the PFC response in nude mice (Table 12). The results of this experiment suggest that (a) thymus-derived lymphocytes are required to obtain ATS-induced enhancement, and (b) enhancement is not the result of a stimulatory effect of ALS upon B cells. If regulatory T cells exert only a negative influence on the PVP immune response, nude mice which lack functional T cells might be expected to respond in a manner similar to ATS-treated normal mice. However, nude mice and normal mice produce similar numbers of PVP-specific PFC in response to an optimally immunogenic dose of PVP. This suggests that nude mice lack not only a subpopulation of T cells that normally exerts a negative influence on the magnitude of the immune response but also a T cell subpopulation that can amplify the PFC response to PVP.

Table 12. Effect of Treatment with ATS on the Magnitude of the PFC Response to PVP in Nude and Normal BALB/c Mice

Mice	Treatment of Mice	PVP-Specific PFC[a]	
		Spleen	10^6
Normal	NRS, 0.3 ml	4.123 ± 0.239[b]	1.954 ± 0.279
		(13,274)[c]	(90)
Normal	ATS, 0.3 ml	5,000 ± 0.140	2.871 ± 0.125
		(100,000)	(743)
Nude	NRS, 0.3 ml	3.818 ± 0.304	1.713 ± 0.293
		(6,577)	(52)
Nude	ATS, 0.3 ml	3.826 ± 0.298	1.744 ± 0.322
		(6,699)	(56)

[a] Numbers of PVP-specific PFC per spleen and per 10^6 spleen cells detected 5 days after immunization with 0.25 µg PVP and injection with ATS or NRS. ATS, Microbiological Associates Lot 14580.

[b] Log_{10} ± standard deviation for 10 similarly treated mice.

[c] Geometric mean.

Effect of Adult Thymectomy on the PFC Response to PVP and ATS-Induced Enhancement

To investigate the effect of another method of T cell depletion, normal mice 6-7 weeks old were thymectomized. Eight weeks following thymectomy groups of age matched normal control mice, sham thymectomized, and thymectomized mice were given an optimally immunogenic dose of PVP with or without ATS. The magnitude of the PFC response was assessed 5 days after immunization. The results (Table 13) show that depletion of T cells by ATS treatment resulted in about a 5-fold enhancement of the PVP response and removal of the thymus caused a 2.5-fold increase in the number of PVP-specific PFC when compared with age-matched non-thymectomized, non ATS treated controls. Sham thymectomy alone, also, induced a modest increase in the PFC response to PVP. ATS treatment of adult thymectomized mice resulted in the greatest degree of enhancement observed.

These results are consistent with the hypothesis that depletion of T cells by either ATS or adult thymectomy or both removes a population of thymus-derived cells which normally exerts a negative influence on the magnitude of the immune response to PVP. The depletion of suppressor T cells allows the expression of a subpopulation of T cells which amplifies (amplifier T cells) the immune response to PVP.

Table 13. Effect of Adult Thymectomy on the PFC Response to PVP and ATS-Induced Enhancement

Group	No. of Mice	Treatment	ATS	PFC/Spleen ± SD[b]
A	6	None	-	19,050 ± 5,870
B	8	None	+	106,344 ± 43,113
C	6	Sham Thymec.	-	30,460 ± 10,018
D	8	Thymectomized	-	51,266 ± 15,941
E	6	Thymectomized	+	133,836 ± 84,184

[a]Eight weeks following adult thymectomy, sham thymectomy, or no treatment, mice were immunized with 0.25 µg of PVP and in some groups given an i.v. injection of 0.3 ml of a 1:2 dilution of ATS which had been prepared in this laboratory. The number of PVP-specific PFC was determined 5 days following immunization.

[b]Arithmetic mean PFC/spleen ± SD for 6-8 similarly treated mice.

The increase induced following sham thymectomy may be due to the induction of a stress related involution of the thymus (61) again resulting in a partial depletion of a subpopulation of T cells.

PVP-Specific PFC Response in Nude Mice Given BALB/c Thymus or Bone Marrow Cells

Because nude mice lack regulatory T cells, it was possible that the injection of normal BALB/c thymocytes into nude mice would supply the missing suppressor T cells resulting in decreased PFC responses to PVP when compared to untreated nude mice. To test this possibility, 15 nude mice were given an optimally immunogenic dose of PVP and then divided into 3 equal groups. Group A was not treated further, group B was given 2×10^8 BALB/c thymus cells, and group C was given 1.5×10^8 BALB/c bone marrow cells. The number of PVP-specific PFC was determined 5 days following immunization. Contrary to expectations, the results (Table 14) demonstrate that the injection of normal thymus cells into nude mice led to about a 2-fold enhancement of the response to PVP rather than a suppression. As expected, the injection of bone marrow cells resulted in a 3-fold enhancement of the response due probable to the addition of increased numbers of B cells capable of responding to PVP.

The failure of transferred thymocytes to suppress the response to PVP in nude mice could have been due to a number of reasons: (a) thymocytes may need to be injected prior to immunization to allow time

Table 14. PVP-Specific PFC Response in Nude Mice Given BALB/c Thymus or Bone Marrow Cells[a]

Group	Thymus	Bone Marrow	PVP-Specific PFC/Spleen[b]
A	--	--	4,713
B	2×10^8	--	10,925
C	--	1.5×10^8	14,050

[a]Nude mice were given an i.v. injection of 2×10^8 BALB/c thymus or bone marrow cells in a volume of 0.5 ml. Immediately following cells transfer all mice were immunized with 0.25 μg of PVP and the number of PVP-specific PFC was determined 5 days later.

[b]Arithmetic mean PFC/spleen for 5 similarly treated mice.

for these cells to mature into suppressor T cells. (b) The enhancement observed following injection of thymocytes could have been due to an allogenic effect since the nude mice used in these experiments were not congenic with BALB/c mice. (c) The thymocytes could have been contaminated with B cells derived from parathymic lymph nodes. (d) Nude mice may lack some essential thymic factor necessary to properly mature thymocytes. I was not able to distinguish among these possibilities.

PFC Response and ATS-Induced Enhancement to PVP in Nude Mice Implanted with Thymus Glands

The implantation of syngeneic thymus glands in nude mice results in almost normal thymus-dependent immune responses (62). I wished to determine if thymus gland-grafted nude mice (nude-TG) would be able to mount enhanced PFC responses to PVP following ATS treatment. Neonatal BALB/c thymus glands were implanted under the renal capsule of nude mice. Eight weeks following grafting groups of BALB/c, nude, and nude-TG mice were immunized or immunized and treated with ATS. The number of PFC was determined 5 days following immunization. The results (Table 15) show that BALB/c mice treated with ATS had 5-fold higher PFC responses than untreated BALB/c mice whereas nude mice treated with ATS had approximately the same number of PVP-specific PFC as untreated nude mice. Unexpectedly, nude-TG given an optimally immunogenic dose of PVP had PVP-specific PFC responses which were 7-fold higher than the responses of similarly treated normal or nude

Table 15. Effect of ATS Treatment of BALB/c, Nude, or Nude-TG on the PFC Response to PVP[a]

Group	No. of Mice	ATS Treatment	PVP-Specific PFC/Spleen ± SD[b]
BALB/c	6	-	14,921 ± 5,560
BALB/c	6	+	79,491 ± 26,028
Nude	6	-	13,304 ± 8,889
Nude	6	+	17,423 ± 7,068
Nude-TG	7	-	107,839 ± 88,083
Nude-TG	7	+	123,271 ± 66,015

[a]Nude mice were grafted with a single neonatal BALB/c thymus gland under each renal capsule. Eight weeks following grafting groups of BALB/c, nude, and nude-TG mice were immunized with 0.25 µg of PVP and in some groups given an i.v. injection of 0.3 ml of a 1:2 dilution of ATS prepared in this laboratory. The number of PVP-specific PFC was determined 5 days following immunization.

[b]Arithmetic mean PFC/spleen ± SD for 6-7 similarly treated mice.

mice. Nude-TG immunized and treated with ATS had even higher PFC responses.

Effect of ATS Treatment on the Magnitude of the PFC Response to PVP and BE in BALB/c, Nude, and Nude-TG Mice

Groups of normal, nude, and nude-TG were doubly immunized with PVP and BE. Half of the animals in each group were treated with ATS. The magnitude of the PFC response to PVP and BE was determined 5 days following immunization. The results (Table 16) show that ATS treatment of BALB/c mice caused a 5-fold increase in the response to PVP and a 2-fold decrease in the number of BE-specific PFC. Nude mice responded well to PVP but had less than 1% of the BE response of normal mice; ATS treatment of nude mice did not increase the magnitude of the PFC response to PVP or BE. Nude-TG had 7-fold enhanced PVP-specific PFC responses and BE-specific PFC responses which were similar to BE responses in normal mice. As expected, ATS treatment of nude-TG caused a further increase in the PFC response to PVP. Surprisingly, ATS treatment of nude-TG did not affect the magnitude of the BE-specific PFC response.

These results confirm previous findings: (a) Nude mice respond well to PVP, a thymus-independent antigen, but respond very poorly to the thymus-dependent antigen, BE. (b) ATS treatment of normal mice results in enhanced PVP responses and decreased BE responses while ATS

Table 16. Effect of ATS Treatment of BALB/c, Nude, or Nude-TG on the PFC Response to PVP and BE[a]

Group	Treatment	PFC/Spleen[b]	
		PVP-Specific	BE-Specific
BALB/c	PVP + BE	8,542 ± 2,815	46,458 ± 32,908
BALB/c	PVP + BE + ATS	43,854 ± 11,611	25,958 ± 2,499
Nude	PVP + BE	4,500 ± 2,165	120 ± 54
Nude	PVP + BE + ATS	3,267 ± 1,716	117 ± 141
Nude-TG	PVP + BE	56,375 ± 14,234	24,725 ± 9,841
Nude-TG	PVP + BE + ATS	109,000 ± 55,350	29,675 ± 5,515

[a] Nude mice were grafted with a single neonatal BALB/c thymus gland under each renal capsule. Eight weeks following grafting groups of BALB/c, nude, or nude-TG mice were immunized with 0.25 μg of PVP and 0.2 ml of a 10% suspension of BE and in some groups mice were given an i.v. injection of 0.3 ml of a 1:2 dilution of ATS which had been prepared in this laboratory. The number of PVP-specific or BE-specific PFC was determined 5 days following immunization.

[b] Arithmetic mean PVP-specific or BE-specific PFC/spleen ± SD for 5 similarly treated mice.

treatment of nude mice does not alter the PFC response to PVP. (c) Nude-TG have markedly increased responses to PVP.

Also, the results presented here demonstrate that nude-TG have elevated PVP-specific PFC responses when helper T cell function appears almost normal as assessed by the response to BE. These results can be explained by proposing a differential repair of thymus function in nude mice implanted with thymus glands. The activities of helper T cells and T cells amplifying the response to PVP appear to be nearly normal while the activity of cells which would normally exert a negative influence on the magnitude of the PVP response is greatly diminished.

PVP and SSS-III-Specific PFC Responses in BALB/c, Nude, and Nude-TG Mice

To determine if nude-TG would support enhanced antibody responses to another helper T cell independent antigen, groups of normal, nude, and nude-TG mice were given a mixture of an optimally immunogenic dose of PVP and SSS-III. The number of PVP and SSS-III-specific PFC was determined 5 days later. Normal PFC responses to both antigens were found in BALB/c and nude mice. Nude-TG, on the other hand, had increased numbers of PVP-specific PFC compared to either BALB/c or nude mice. But, in the same mice the magnitude of the PFC response to SSS-III was not altered (Table 17). These results appear to be similar to results obtained in adult thymectomized mice in which the PFC

Table 17. PVP and SSS-III-Specific PFC Responses in BALB/c, Nude, and Nude-TG Mice[a]

Group	No. of Mice	Immunization	PFC/Spleen SD[b] PVP-Specific	SSS-III-Specific
BALB/c	3	PVP + SSS-III	13,450 ± 2,212	9,463 ± 5,722
Nude	5	PVP + SSS-III	5,500 ± 2,312	13,225 ± 11,860
Nude-TG	5	PVP + SSS-III	49,250 ± 14,034	9,725 ± 3,875

[a]Nude mice were grafted with a single neonatal BALB/c thymus gland under each renal capsule. Eight weeks following grafting groups of BALB/c, nude, and nude-TG mice were given an i.v. injection of a mixture of 0.25 μg of PVP and 0.5 μg of SSS-III. The number of PVP-specific and SSS-III-specific PFC were determined 5 days following immunization.

[b]Arithmetic mean PVP-specific or SSS-III-specific PFC/spleen ± SD for 5 similarly treated mice.

response to PVP is increased (Table 13, 37) while the PFC response to SSS-III is not (47). T-1 cells are rapidly depleted following adult thymectomy (60) and it is possible that these cells are at least in part responsible for suppressor T cell activity directed against antibody responses to PVP but not SSS-III. Therefore, nude-TG may have lowered numbers of T-1 cells present in their spleens.

Effect of Thymosin Treatment on the PVP-Specific PFC Response in BALB/c, Nude, and Nude-TG Mice

Previous studies (27,60) suggest that short-lived suppressor T cells having the attributes of T-1 cells depend upon the presence of an intact thymus gland or, alternatively, the soluble products produced by the thymus. Recently, a thymic factor derived from pig blood has been shown to restore short-lived suppressor T cell function in NZB mice (63) and treatment of adult thymectomized mice with calf thymosin allowed the development of suppressor T cells following an injection of picryl sulfonic acid (64).

In light of these results, the effect of thymosin treatment on the PVP-specific PFC response of nude-TG mice was tested. Groups of BALB/c, nude, and nude-TG mice were given 9 i.p. injections of 500 µg to thymosin fraction V, spaced over 3 weeks time. One day following the last injection of thymosin all mice were given an optimally immunogenic dose of PVP and the magnitude of the PVP-specific PFC response was determined 5 days later.

The results presented in Table 18 show that thymosin treatment did not affect the magnitude of the PVP-specific PFC response of BALB/c or nude mice but nude-TG mice treated with thymosin had PVP-specific PFC responses which were less than half of the PFC responses in none treated nude-TG and were similar to responses found in normal BALB/c mice. These results are consistent with the hypothesis that nude-TG have diminished T-1 suppressor cell activity 8 weeks following implantation of neonatal BALB/c thymus glands under their renal capsule. This suppressor T cell activity can be restored by treatment of these mice with thymosin, a thymus hormone derived from calf thymus.

PVP-Specific PFC Response of BALB/c Mice Implanted with Syngeneic Thymus Glands

Does the nude mouse provide a unique environment which causes thymus function to mature in the fashion previously described, or will similar results occur if normal mice are implanted with syngeneic thymus glands? In an attempt to answer this question neonatal BALB/c thymus glands were implanted under the renal capsule of BALB/c mice. Eight weeks later the BALB/c gland grafted mice (BALB/c-TG) and untreated control mice were given an optimally immunogenic dose of PVP. Five days following immunization the mice were killed, examined for the presence of visible thymus glands under the renal capsule, and the number of splenic PVP-specific PFC determined.

Table 18. Effect of Thymosin Treatment on the PVP-Specific PFC Response in BALB/c, Nude, and Nude-TG Mice[a]

Group	No. of Mice	Treatment	PVP-Specific PFC/Spleen	SD[b]
BALB/c	5	-	17,775 ±	9,745
BALB/c	5	Thymosin	21,713 ±	7,738
Nude	5	-	9,550 ±	4,564
Nude	4	Thymosin	10,031 ±	8,200
Nude-TG	3	-	52,083 ±	9,382
Nude-TG	5	Thymosin	24,500 ±	12,243

[a]Nude mice were grafted with a single neonatal BALB/c thymus gland under each renal capsule. Eight weeks following grafting half the mice in groups of BALB/c, nude, and nude-TG mice were injected i.p. with 500 µg of thymosin fraction 5 on Monday, Wednesday, and Friday for 3 weeks. One day after the last thymosin treatment, all mice were immunized with 0.25 µg of PVP. The number of PVP-specific PFC was determined 5 days following immunization.

[b]Arithmetic mean PFC/spleen ± SD for 3-5 similarly treated mice.

The results of Table 19 show that BALB/c-TG had 3-fold higher PFC responses compared to normal BALB/c controls. Although not as dramatic, these results do resemble those obtained in nude-TG mice.

However, at necropsy the implanted thymus gland were not visible in BALB/c-TG; in contrast, thymus glands were easily visible under the renal capsule of nude-TG in previous experiments. Also, it is possible that the surgical manipulation of these mice could have caused a stress induced involution of thymus tissue resulting in increased PVP responses. Thus, it is important to include sham operated control mice in experiments involving potentially stressful situations. For this reason and the difficulty in finding the implanted glands at necropsy, this experiment is being repeated including the appropriate controls.

Treatment of Mice with Hydrocortisone Acetate

Metcalf (49) and Dukor et al. (48) have described a characteristic sequence of events which occurs in thymus tissue following grafting. Immediately following the grafting of neonatal thymus glands to adult syngeneic mice, widespread death of cells occur in the grafted tissues. After an initial massive necrosis involving all but a narrow rim of cells in the periphery of the graft, the organ collapses inwards around surviving groups of epithelial and reticulum cells and some cortical lymphoid cells. Mitotic activity recommences in the surviving lymphoid cells and regeneration takes place; normal thymus

Table 19. PVP-Specific PFC Response of BALB/c Mice Implanted with Syngeneic Thymus Glands[a]

Group	No. of Mice	Treatment	PVP-Specific PFC/Spleen±SD[b]
A	5	None	10,595 ± 4,284
B	13	Thymus gland grafted	36,450 ± 15,944

[a] BALB/c mice were grafted with a single neonatal BALB/c thymus gland under each renal capsule. Eight weeks following grafting groups of normal BALB/c and thymus gland grafted BALB/c mice were immunized with 0.25 μg of PVP. The number of PVP-specific PFC was determined 5 days following immunization.

[b] Arithmetic mean PFC/spleen ± SD for 5-13 similarly treated mice.

architecture is restored by 2 to 3 weeks following grafting. Initially the lymphoid elements of the grafted thymus are of donor origin but these gradually become replaced so that by 3 to 4 weeks post grafting, the gland is composed of an epithelial and reticulum cell spongework of donor origin with proliferating lymphoid cells of host origin.

A similar series of events appears to occur in the thymus of normal mice after the administration of hydrocortisone acetate (HC). Ishidate and Metcalf showed (65) that death of small lymphocytes began in the thymus within 3 hours following the injection of 1.0 mg of HC to C3H mice. By 24 hours the cortex was almost completely denuded of identifiable lymphocytes. The reticular framework of the cortex collapsed around the medulla, in which the changes were less marked with some lymphocytes surviving the effects of the cortisone. By day 3, regeneration of small lymphocytes had commenced and by day 7, mitotic activity had re-appeared in the subcapsular region of the cortex and an increased number of cells was present in the cortex. Ten days following injection of HC normal thymus morphology was restored although the size of the organ was smaller and more mitotic figures were visible compared to normal thymus glands.

Both thymus grafting and cortisone treatment result in severe damage to the thymus gland followed by regeneration and restoration of normal thymus morphology. Previous experiments (Tables 15-18) have

demonstrated the presence of enhanced PFC responses to PVP in thymus gland grafted nude mice possibly due to a differential maturation of thymus function. In light of the previous descriptions, it was of value to determine if HC induced thymus involution and subsequent regeneration would lead to enhanced PFC responses to PVP. Accordingly, groups of BALB/c mice treated either 50 or 60 days previously with 2.5 mg HC were given an optimally immunogenic dose of PVP. The magnitude of the PVP-specific PFC response was determined 5 days later.

The results presented in Table 20 show that mice treated 50 or 60 days previously with 2.5 mg HC have only slightly increased numbers of PFC to PVP. There was, however, a very large range of PFC values in the two treatment groups as evidenced by the large standard deviations reported. For example, in the group receiving HC 60 days previous to immunization, the PFC values ranged from 15,825 to 83,425 PFC/spleen. It seems likely, therefore, that HC treatment affects individual mice differently, at least in respect to their ability to respond to PVP. Alternatively, it is possible that the time between treatment and immunization was either too long or too short to observe optimal results. Thus, the large range in values might have occurred because the mice were immunized on the lower or upper limit of the effects of the HC treatment. Also, Rotter and Trainin (37) have shown that HC resistant thymus cells were much more effective than normal thymus cells in reducing enhanced PFC responses to PVP in T cell depleted mice. Thus, it

Table 20. PVP-Specific PFC Response of BALB/c Mice Treated With Hydrocortisone Acetate[a]

Group	No. of Mice	Treatment	PVP-Specific PFC/Spleen ± SD[b]
A	5	None	19,765 ± 6,988
B	4	HC (-50 days)	28,143 ± 17,640
C	6	HC (-60 days)	37,183 ± 27,081

[a] BALB/c mice were given a single i.p. injection of 2.5 mg of hydrocortisone acetate. Fifty or 60 days later groups of normal and HC-treated mice were immunized with 0.25 µg of PVP. The number PVP-specific PFC was determined 5 days following immunization.

[b] Arithmetic mean PFC/spleen ± SD for 5 similarly treated mice.

is possible that T cells which can act to suppress the response to PVP can survive HC treatment and are able to repopulate the damaged thymus in a nearly normal manner.

Treatment of PVP-Immunized Mice with Concanavalin A

Concanavalin A (Con A), a plant lectin, nonspecifically activates a larger percentage of T lymphocytes but not B lymphocytes. These mitogen actived T cells express many of the immunological activities characteristic of antigen-actived cells. Rich and Pierce (66) showed that T cells from mice injected 24 hr previously with 150 µg Con A suppressed PFC response by normal spleen cells in vitro. If the immune response to PVP can be regulated by activated suppressor or amplifier T cells, the injection of Con A into PVP-immunized mice should modify the PFC response to this antigen. Using a protocol suggested by Dr. P. J. Baker (personal communication) the previous hypothesis was tested. Fifteen BALB/c mice were given an optimally immunogenic dose of PVP and then divided into three groups. The mice in group A received no further treatment, the mice in group B were given 300 µg of Con A i.v. immediately following immunization, and the mice in group C were given 300 µg of Con A 2 days following immunization. The magnitude of the PFC response was determined 5 days following immunization for all mice.

The results (Table 21) show that mice injected with Con A and immunized with PVP on the same day had 8.6-fold fewer PFC than did non-Con A treated controls whereas mice treated with Con A 2 days following immunization had 4.8-fold higher PFC values than untreated controls. In light of previous extensive studies concerning the effects of Con A activated T cells on in vitro immune response (27), the results of this preliminary experiment could be explained by proposing that Con A nonspecifically activates T cells which can either suppress or amplify the immune response to PVP. However, much more evidence must be collected before this proposal can be accepted.

Effect of Prior Immunization with PVP on the PVP-Specific PFC Response

During the course of these studies it was useful to determine whether mice could produce secondary immune responses to PVP. The first of such experiments was performed to determine the effect of priming with an optimally immunogenic dose of PVP on the PFC response of mice following a second, optimally immunogenic dose of PVP. Mice were primed with 0.25 μg PVP and challenged 2 weeks later with 0.25 μg of PVP; the number of PVP-specific PFC was determined 5 days following challenge. The response of primed, immunized mice (Table 22, Group B) was 5.5-fold lower than the response of mice which received a single, immunogenic dose of antigen (Table 22, Group A).

Table 21. Effect of Con A Treatment on the Magnitude of the PFC Response of PVP[a]

Group	No. of Mice	Treatment	PVP-Specific PFC/Spleen	SD[b]
A	5	None	17,655 ± 8,130	
B	5	Con A (day 0)	2,045 ± 718	
C	5	Con A (day 2)	85,745 ± 53,462	

[a]BALB/c mice were immunized with 0.25 µg of PVP and at the same time the mice in group B were given in i.v. injection of 300 µg of Con A. Two days following immunization the mice in group C were given an i.v. injection of 300 µg of Con A and the number of PVP-specific PFC was determined 5 days after immunization.

[b]Arithmetic mean PFC/spleen ± SD for 5 similarly treated mice.

Table 22. PFC Response of Mice of PVP Following Primary and Secondary Immunization

Group	PVP Immunization[a]		PFC/Spleen	
	Day 0	Day 14	Direct	Direct + Facilitated
A	-	+	4.334 ± 0.103[b]	4.322 ± 0.123
			(21,581)[c]	(20,970)
B	+	+	3.607 ± 0.259	3.695 ± 0.204
			(4,045)	(4.957)

[a]The mice in group B were given 0.25 µg of PVP on day 0. On day 14 all mice were given 0.25 µg PVP and the number of direct and facilitated PVP-specific PFC was determined 5 days later.

[b]Log_{10} PFC/spleen ± SD for 5 similarly treated mice.

[c]Geometric mean.

Previous results (Table 4, Fig. 6) indicated that mice immunized with PVP produced only IgM PFC 5 days following primary immunization. Similarly, in the present experiment the numbers of direct and direct plus facilitated PFC were similar after a primary injection (Table 22) and after a secondary immunization (Table 22, Group B), eliminating the possibility that secondary immunization caused a shift in the immunoglobulin class of antibody producing cells.

These results seem to contradict a previous report (55) which indicated that prior immunization with PVP did not affect the serum antibody level following a second injection of antigen. However, it was possible that differences in doses, time between priming and challenge immunization, or assay methods could have caused the differing results. I, therefore, thought it useful to study the characteristics of the unresponsive state produced in mice by prior immunization with PVP.

Effect of Varying the Priming Dose on the Magnitude of the PFC Response to PVP Following a Second, Optimally Immunogenic Dose

The next experiment was performed to determine if varying the priming dose would affect the magnitude of the PVP-specific PFC re- following a second, optimally immunogenic dose of PVP. Groups of mice were primed with PVP doses ranging from 2.5×10^{-6} µg to 2.5 µg and challenged 3 days later with an optimally immunogenic dose of PVP (0.25 µg). The magnitude of the PVP-specific PFC response was assessed

5 days following challenge. The responses obtained were compared to the response elicited by an optimally immunogenic dose of PVP (0.25 μg) in unprimed mice (control group). The results (Figure 7), presented as per cent of control PFC values, show that, with the exception of the lowest priming dose (2.5×10^{-6} μg), all of the doses of PVP used for priming reduced the magnitude of the PFC response to the second, optimally immunogenic dose of antigen. The degree of paralysis was dependent upon the magnitude of the priming dose over the lower dose range. Priming doses considerably below detectable immunogenic levels were able to markedly reduce the magnitude of the PFC response to a subsequent, optimal dose of PVP. Thus, in comparison to the control group, mice primed with 2.5×10^{-3} μg, 2.5×10^{-4} μg and 2.5×10^{-5} μg of PVP had 72, 66 and 41% fewer PVP-specific PFC, respectively. Maximal paralysis (10% of control response) was observed in mice primed with 0.025 μg of PVP. Priming doses greater than 0.025 μg resulted in approximately the same degree of paralysis at a level slightly less than the maximum. Because maximal paralysis was observed in mice primed with 0.025 μg of PVP, this priming dose was used in the following experiments dealing with the induction of low-dose paralysis of PVP.

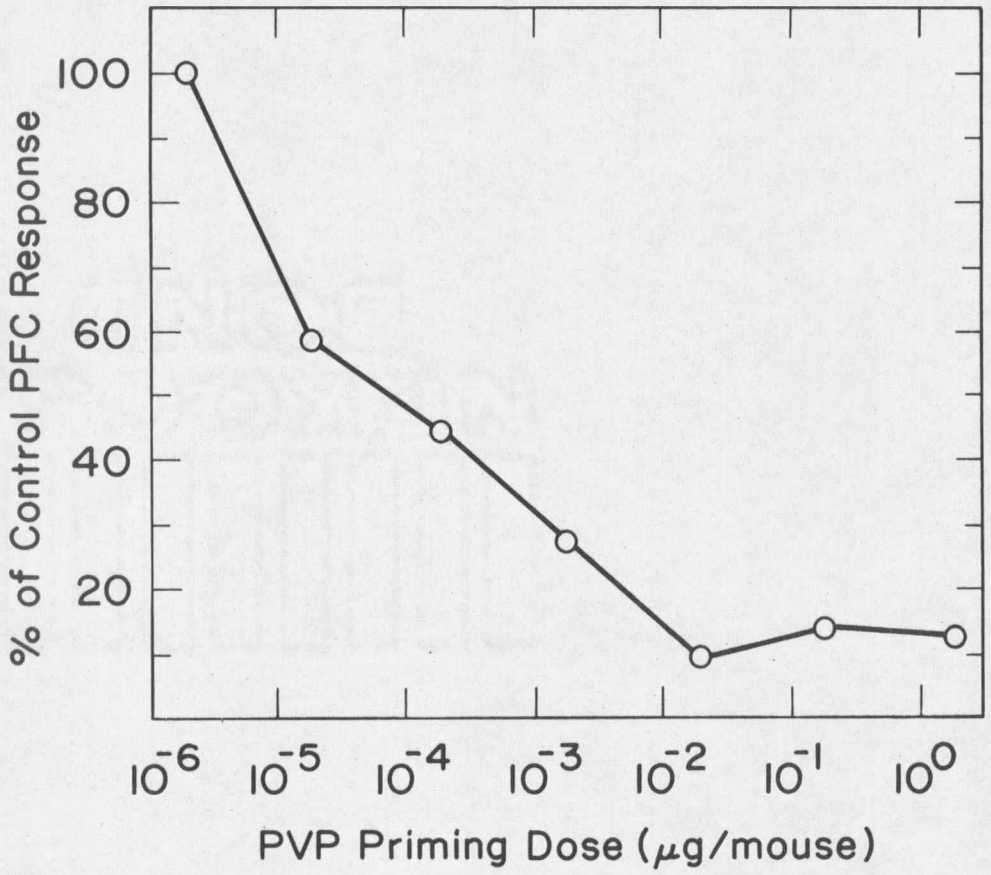

Figure 7. Effect of varying PVP Priming Doses on the PVP-Specific PFC Response Following a Second, Optimally Immunogenic Dose of PVP. Mice were primed with varying amounts of PVP and challenged 3 days later with 0.25 μg of PVP. The number of PVP-specific PFC was determined 5 days following challenge. Results, determined from 6 to 8 mice per group, are expressed as per cent of control PFC/spleen response. The magnitude of the control PFC response was determined from mice which were challenged only (0.25 μg of PVP).

Effect of Varying the Time Between PVP-Priming and Challenge on the Secondary PFC Response

It was possible that the amount of time between PVP priming and challenge immunization would greatly affect the magnitude of the secondary PFC response. To test this possibility, groups of mice of equal age were primed with PVP (0.025 μg) on various days before challenge immunization with an optimally immunogenic dose of PVP. The number of PVP-specific PFC was determined 5 days following challenge. The results (Figure 8) indicate that the unresponsive state can be observed as early as one day following priming and that maximal unresponsiveness is attained 2 days following priming. The unresponsive state is then maintained at approximately the maximal level through day 20 following priming. By day 30 after priming the magnitude of the PVP-specific PFC response returns to a level equivalent to a normal primary response and, interestingly, by day 60 following priming is 2.2-fold higher than the primary response.

Specificity of PVP-Induced Paralysis

The previous experiments demonstrated that prior administration of PVP resulted in a marked reduction in the magnitude of the PFC reponse to PVP following a second, optimally immunogenic dose of PVP. The following 2 experiments were performed to determine the specificity of the unresponsive state. In the first, the effect of PVP-priming on the magnitude of the PFC response to a thymus-dependent