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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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

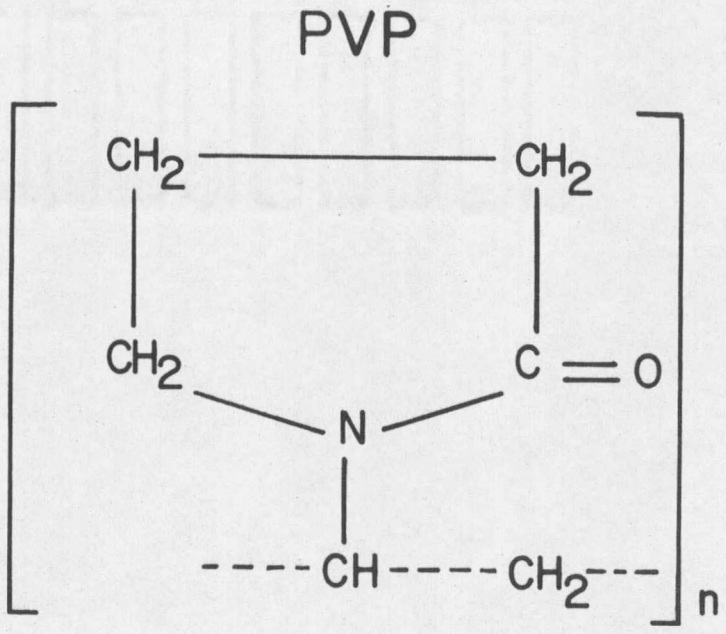

Figure 1. Diagrammatic Representation of Polyvinylpyrrolidone.