1976

Temporal effects of various immunosuppressive drugs on the immune response

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TEMPORAL EFFECTS OF VARIOUS IMMUNOSUPPRESSIVE DRUGS ON THE IMMUNE RESPONSE

by

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B.S., Nebraska Wesleyan University, 1971

Presented in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

UNIVERSITY OF MONTANA

1976

Chairman, Board of Examiners

Dean, Graduate School

Date June 10, 1976

Swanson, Priscilla A., M.S. June 1976 Microbiology

Temporal Effects of Various Immunosuppressive Agents on the Immune Response (89 pp)

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Immunosuppressive agents, those which suppress or prevent various phases of the immune response, have been used experimentally to analyze the basic cellular and molecular events involved in normal immune responses and have been applied clinically during therapeutic programs for organ transplantation, autoimmune disease, and malignancy. The immune mechanism encompasses several overlapping stages, primarily the afferent arm or induction of immunity and the efferent arm or expression of immunity. In addition, the immune response consists of two distinct cell compartments. Thymus-derived lymphocytes, T cells, differentiate into cells functioning in cellular immunity while bone-marrow-derived lymphocytes, B cells, are activated to produce and secrete antibody. With regard to the type of drug, dosage, schedule, and route of administration, any of the levels of the immune response are subject to suppression.

In this study four drugs were investigated superficially in an attempt to understand the interaction of drugs and the immune response and to define the lymphoid cell population(s) most susceptible to suppression. Emphasis was placed on hydrocortisone acetate while azathioprine, niridazole, and methotrexate were studied in less detail. Immunosuppressive effects on induction and expression of cellular immunity were accomplished by assaying the delayed hypersensitivity (DH) response of drug-treated animals to the chemical sensitizing agent oxazolone. Suppression of humoral immune induction was measured by antibody production of rabbit red blood cells and by spleen B cell levels assayed by immunofluorescence. Subsequent Friend Disease Virus infection of drug-treated mice was also used to determine the presence of functional B cells.

Treatment with these four drugs resulted in immune suppression, but variations in degree, duration, and susceptible cell populations were noted. Hydrocortisone acetate affected both cellular and humoral immunity but appeared to have the most profound effect on T cells involved in expression of DH and induction of antibody production. Azathioprine and niridazole also caused more pronounced suppression of T cell rather than B cell function as shown by depression of the DH response. However, suppression of DH expression was delayed for several days following azathioprine and niridazole treatment whereas cortisone suppressed the DH response immediately. Methotrexate, on the other hand, suppressed the B-cell compartment of the immune response. Cortisone, azathioprine, and niridazole appeared to exert their immunosuppressive effect by altering antigen recognition,either due to defective antigen receptors or unavailable lymphocytes, whereas methotrexate apparently suppressed lymphocyte proliferation.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. Richard N. Ushijima and Dr. Carl L. Larson for their encouragement and guidance throughout my studies at the University of Montana.

In addition, I extend thanks to the members of my committee, Drs. J. Coe, R. A. Faust, and K. Watson for their advice and assistance concerning this research and thesis.

Thanks are extended to Kathy Kelly, Jeanine Green, and Judy Sanderson for offering their time and expertise to teach me many of the techniques involved in this study.

Finally, I would like to acknowledge the Burroughs Wellcome Co. and the American Cyanamid Co. for their gifts of azathioprine and methotrexate respectively.

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Chapter 1

INTRODUCTION

Regulation and control of immune responses occupies a major area of scientific research. Agents which suppress or prevent immunological responses, i.e. immunosuppressive agents, aid in dissecting basic immune mechanisms and are applied clinically to transplant recipients and to patients with autoimmune diseases, hormonal imbalance, malignancy, and hypersensitivity conditions. The effects of these agents range from indiscriminate bombardment of the immune mechanisms to specific effects directed at "target areas" (51).

An understanding of the mechanisms of action of various chemical and physical agents might enable manipulation to remove undesirable immune responses without damaging the vital defenses of the host. However, it must be kept in mind that immunosuppressive agents are as heterogeneous and complex as the immune reactions which are altered, although several basic considerations apply to all groups: heterogeneity of lymphoid cells, species differences, undesirable sequellae of elimination of any component of the immune response, and decreased immunosurveillance resulting in increased threat of malignancy and autoimmune disease.

The immune mechanism consists of several overlapping stages, predominantly the processing of antigen or afferent arm and the eliciting of the immune response or efferent arm (20). Since immuno-

suppressive agents may alter one or more of these processes, a discussion of the immune mechanism follows.

Antigens entering a host are ingested and processed by macrophages facilitating immobilization or concentration of these antigens for easier access to lymphoid cells. Although the immune system is composed of two distinct populations of lymphocytes, these cells originate as precursor bone marrow stem cells capable of responding to antigen under normal conditions.

One type of lymphocyte is known as thymus-derived or T lymphocyte. The stem cells migrate to the thymus gland, develop characteristic surface antigens, and differentiate into cells which function in cellular immunity. The T lymphocytes are responsible for defense against viruses, fungi and intracellular pyogenic bacterial pathogens, delayed allergic reactions, and surveillance and destruction of malignant cells. These lymphocytes proliferate in the cortical area of the thymus then shift to the medullary region from where they enter the circulatory system and peripheral lymphoid tissues as competent T cells. During maturation TL isoantigens and the theta isoantigen arise on the cell surface (16). The theta antigen is retained on cells throughout their circulation and constitutes a T cell marker. Because T cells are continually circulating and recirculating, the population may be depleted by such methods as thymectomy, thoracic duct drainage, or chemotherapy, which will be important in later considerations of immunosuppressive drugs.

The second component of the lymphoid system consists of Bursa-equivalent derived cells or B lymphocytes, which differentiate into

cells functioning in humoral immunity. Following antigenic stimulation through the mediation of a macrophage, B cells differentiate into immunocompetent plasma cells and produce and secrete immunoglobulins. A single clone of B cells secretes only one type of antibody and may be identified by its surface immunoglobulin. These cells appear to be localized in the medullary cord, central lymphoid follicles, and peripheral cortical regions of the lymph nodes, the splenic red pulp and peripheral white pulp, and the subepithelial lymphoid follicles (51). Table 1 gives some characteristics of T and B lymphocytes.

Antibody production for certain antigens requires interactions of the T and B-cell compartments. Certain T lymphocytes function as "helper" cells and are activated by antigen. These activated T cells interact with B cells, which in turn produce circulating antibody. T cell - B cell cooperation may or may not be required for antibody synthesis. Antigens such as endotoxin, pneumococcal polysaccharide, and polyvinylpyrrolidone are T cell independent suggesting that B cells probably have antigen recognition sites, while sheep red blood cells (SRBC) and serum protein antigens are T cell dependent. Interactions of the two components tend to amplify the immune response.

Immunosuppressive drugs are used to study the functions of normal and undesirable immune responses. Variations in the type of drug, dosage, and route of administration aid in defining and dissecting induction and production phases of the immune response, delayed hypersensitivity and nonspecific inflammatory responses, drug induced immunological tolerance, and homograft rejection. Manipulation of induction and production of the immune response leads

Table 1. Comparison of T and B lymphocytes (9).

Properties	B cells	T cells
Tissue origin	Bursa of Fabricius (in birds) or bone marrow	Thymus
Cell surface antigen	H-2 transplantation antigen	Theta TL
Antigen receptors	Surface immunoglobulins	Unknown
Hapten-antigen recognition	To hapten	To carrier antigens
Tissue distribution	High in spleen, low in lymphatic tissue and blood	High in thoracic duct, lymph nodes, and blood
Functions	Secretion of antibody	Helper function, effector cell for cell-mediated immunity
Complement receptors	Binds C_3	None known
Mitogen susceptibility	Pokeweed Lipopolysaccharide	Phytohemagglutinin Conconavoline A Pokeweed
Steroid susceptibility	Resistant	Sensitive
X-irradiation susceptibility	Sensitive	Resistant
Tolerance	Occurs late, of short duration	Occurs early, persists

to an understanding of the multicellular nature of antibody responses and the differential susceptibility of different cell populations to drug treatment.

The inductive phase of the antibody response is that time between contact of antigen with the host and antibody production. Since low levels of antibody may be beyond detection, the presence of an inductive phase was questioned. Sterzl (87) injected newborn rabbits with spleen cells and antigen. When administering 6-mercaptopurine (6-MP), which substitutes for purines in nucleic acids, with or shortly following the spleen cell transfer, he noted a marked decrease in antibody production. If donor plasma cells secreting antibody were used, suppression did not occur, suggesting that the drug interferes with nucleic acid synthesis in uncommitted lymphocytes. Gnotobiotic piglets injected with SRBC's and actinomycin D, which binds to guanine residues, also show no antibody production, whereas control animals develop antibodies in three days (88). Therefore, induction of antibody formation probably depends on RNA synthesis leading to mitosis. In two classic studies by Berenbaum (11, 12) irradiation or busulfan given prior to antigenic stimulation inhibited antibody response to TAB (typhoid - paratyphoid A and B) vaccine in mice whereas methotrexate (MTX), 6-MP, 6-thioguanine (6-TG), or nitrogen mustard inhibited the response if given following the antigenic stimulation. Evidently, immunocompetent cells are sensitive to irradiation and busulfan but become susceptible to other immunosuppressive drugs when differentiated to immunoblasts.

During the productive phase of the primary immune response

when antibody is being secreted, cells are proliferating and mRNA is being produced. When cells in this phase are exposed to metabolic inhibitors, replication is blocked but antibody production is not significantly depressed (89). Sterzl (90) reports that administration of 6-MP during the primary response has little effect on the secondary response, but X-irradiation or cyclophosphamide given during the primary response inhibits the secondary response. Another interesting effect of immunosuppression on the productive phase is that low doses of 6-MP extend IgM production while IgG synthesis is diminished (85).

Turning attention to drug effects on cellular immunity, interpretation of immunosuppressive effects on delayed hypersensitivity depends upon whether suppression is occurring at the level of the immunological mechanism which is specific and has a distinct induction period or on the nonspecific level of the inflammatory reaction. For example, both MTX and cyclophosphamide block development of primary contact sensitivity in the guinea pig (99). However, following passive transfer of sensitized donor lymphoid cells to drug-treated hosts, MTX-suppressed recipients elicit a good delayed hypersensitivity response while cyclophosphamide-suppressed recipients are unable to evoke a delayed reaction. Apparently cyclophosphamide affects both specific and nonspecific phases of cellular immunity and MTX inhibits only cellular proliferation. Some drugs such as 6-MP exert more suppression on delayed hypersensitivity than humoral immunity (15), a fact which indicates that delayed hypersensitivity involves a distinct population of cells rather than simply biochemical changes in the same population of immunologically reactive

cells.

Many immunosuppressive agents are used to retard the cellular changes occurring during homograft rejection, but use of cortisone during kidney transplants is one of the few treatments of clinical significance. Such variability between donor, recipient, and graft exists that interpretation of experimental data becomes difficult. Scothorne (81) finds that cortisone treatment prevents immunoblast formation and development of granulation tissue in rabbits with skin grafts while thalidomide effectively suppresses graft rejection if given to both donor and recipient prior to grafting (102). These drugs probably suppress differentiation of the small lymphocyte, the cell responsible for mediating cellular and humoral immune responses.

The next consideration is the induction of immunologic tolerance by drugs. Tolerance is that process in which a host is unable to respond to a particular antigen but exhibits normal response to other antigens. Tolerance may further be subdivided into those processes in which antigen induces the lack of response and those in which the immune response is present but masked. Early work reports that adult mice infected with lymphocytic choriomengitis (LCM) resist infection if treatment with MTX in initiated (46). Schwartz and Dameshek (84) also show that tolerance to human serum albumin is induced by giving 6-MP with the antigen. Tolerance is specific because antibodies may be produced to bovine gamma globulin given one month later but not to a second dose of human serum albumin. Dose of antigen is extremely critical and not all antigens are tolerogenic. Concerning clinical application of drug

induced tolerance, elimination of antibody secreting cells by drug treatment may increase the population of tolerant cells. In this manner tolerance to a particular antigen may be maintained without continued administration of drug therapy.

Having briefly considered levels of the immune response which are subject to suppression, a review of some of the classes of immunosuppressive drugs will be covered. Immunosuppression may be divided into four general categories: cytotoxic chemical, corticosteroids, irradiation, and biologic agents. The category of cytotoxic chemicals encompasses alkylating agents, folic acid antagonists, antimetobolites of purine bases, analogs of pyrimidine bases, antibiotics, and plant alkaloids.

The cytotoxic agents act nonspecifically by altering basic metabolic functions in cell division, differentiation, and protein synthesis. Their therapeutic usefulness is limited by toxic side effects, especially the indiscriminate suppression of most stages of the immune response. It is interesting to note that depending on dose and timing, enhancement rather than depression of the immune response results. Lance (51) offers possible explanations such as Lebensraum, increased availability of nucleic acids for replication, or perhaps feedback of some immune suppressor mechanism.

One of the earliest alkylating agents, nitrogen mustard, was developed for use in chemical warfare in World War II. This agent produces atrophy of lymphoid tissue and was introduced for treatment of leukemias and lymphomas. Alkylating agents, typified by nitrogen mustard, cyclophosphamide, and busulfan are thought to bind to

electron-rich nucleophilic centers of DNA (especially the N^7 of guanine) and protein in rapidly dividing cells such as the small lymphocytes (83). These agents form cross links between DNA-DNA, DNA-RNA, RNA-RNA, DNA-protein, and RNA-protein resulting in impaired protein synthesis and lymphopenia. Cyclophosphamide affects induction of primary and secondary antibody responses as well as ongoing antibody production if given on the same day as antigen or several days before (1). Macrophage phagocytosis of carbon particles apparently is not inhibited by cyclophosphamide, but proliferation of macrophages and antigen retention in the spleen is decreased (63). Cyclophosphamide also has a profound inhibitory effect on development of delayed hypersensitivity if given prior to the antigen (101). Thus, the effect of alkylating agents is thought to occur early in the induction of the immune response, probably at the level of RNA synthesis. Alkylating agents, though extremely toxic, have been used clinically for treatment of leukemias, multiple myeloma, certain types of carcinomas, and neuroblastoma and experimentally to inhibit development of experimental allergic encephalomyelitis (EAE) and allograft rejection.

The folic acid antagonists, such as aminopterin and amethopterin (methotrexate), have limited use in man due to their extreme toxicity. These agents block the conversion of folic acid to tetrahydrofolic acid by inhibiting the action of the enzyme dihydrofolate reductase. The ultimate effect is inhibition of metabolism of one-carbon units and eventually synthesis of DNA, RNA, and proteins. Methotrexate also blocks differentiation of immunoblasts into small lymphocytes

by selectively depressing dehydrogenase activity (30). Interestingly, the effect of methotrexate may be reversed by administering large doses of folinic acid, thus reducing toxicity while maintaining immunosuppression. Folic acid antagonists cause remarkable suppression of primary and secondary antibody induction, (7S antibody selectively), if given within 24 to 48 hours following antigen or within 72 hours prior (80,98). However, methotrexate is ineffective in suppressing ongoing antibody synthesis. Cell-mediated immune responses such as delayed hypersensitivity to tuberculin and contact sensitivity to oxazolone may also be abolished by folic acid antagonists if the drug were administered within four days following sensitization (101). Reconstitution of drug treated mice with cells of sensitized donors confers delayed type sensitivity. Other cell-mediated immune responses such as graft versus host reaction and allograft rejection are also suppressed by these agents. Folic acid antagonists probably inhibit DNA selectively since immunoblasts develop normally, but development of specific populations of small lymphocytes is blocked even if given after onset of the disease (100). Methotrexate is used experimentally in suppressing LCM infection in mice and EAE in quinea pigs and rabbits. Clinically, methotrexate is accepted for treatment of severe psoriasis, some carcinomas and leukemias, and uveal disease.

The antimetabolites of purine bases, characterized by 6-MP, 6-TG, and azathioprine (Imuran), are synthetic substances which interfere with the biosynthesis of nucleic acids by competing with the nitrogen bases adenine and guanine. Suppression of the 7S

primary antibody response to the intracellular parasite Listeria monocytogenes is accomplished by administering antigen and azathioprine on the same day. However, 19S antibody is not suppressed and may even be enhanced (98). In contrast, azathioprine has no effect on passive transfer of cell-mediated immunity. Mice protected with immune lymphoid cells to Listeria monocytogenes did not succumb to infection when given the drug on the day of immunization or 24 hours prior to passive immunization (97). Apparently, azathioprine is not effective against immunologically active cells but may be restricted to suppression of the inductive phase of immunity. This drug is used extensively in both clinical and experimental suppression of allograft rejection of kidneys, hearts, and livers, especially in combination with other immunosuppressive regimens. Recent use of azathioprine in treatment of systemic lupus erythematosus and glomerulonephritis reveals arrest of the diseases with subsequent suppression of the inflammatory response.

Analogs of pyrimidine bases are mainly used in vitro to study the secondary antibody response. The chemicals 5-bromo-deoxyuridine (BUDR) and 5-fluoro-uracil deoxyriboside (FUDR) are important carcinostatic and virostatic agents which replace uracil and result in the synthesis of abnormal DNA. Cytosine arabinoside and 6-azauridine inhibit DNA polymerase and are incorporated into DNA and RNA. Experiments with FUDR treatment in cancer patients reveal inhibition of primary antibody response in 80%, suppression or delay of secondary response in about 40%, and depression of delayed hypersensitivity to skin sensitizing antigens (60). Cytosine arabinoside affects macrophage differentiation,

inhibits 7S antibody production, mainly *in vitro*, and markedly suppresses cell-mediated immune responses (45).

Use of antibiotics as immunosuppressants is confined mostly to *in vitro* studies. To cite a few, the actinomycins bind to guanine residues of DNA and interrupt DNA directed RNA synthesis. Chloramphenicol inhibits bacterial protein synthesis by binding to mRNA and by suppressing the activity of peptidyl transferase (39). The antibiotics may interfere with the humoral response either by inhibition of antibody synthesis, by interaction with mRNA or tRNA, or by alteration of the genetic code.

The plant alkaloids deserve mention because of their use in combination chemotherapeutic regimens. Such agents as vincristine and vinblastine, derivatives of the periwinkle plant, and colchicine, an extract of the autumn crocus, block spindle formation during metaphase of cell mitosis. In addition colchicine probably inhibits phagocyte digestion of foreign particles. The vinca alkaloids suppress antibody response and DH if given with or after antigenic stimulation (56) whereas colchicine is only effective in suppressing antibody response.

Niridazole (Ambilhar), a new anthelminthic drug introduced in 1964, is used in the treatment of schistosomiasis. The agent is a nitrothiazole derivative 1-(5-nitro-2thiazolyl)-2-imidazolidinone. The drug is apparently concentrated in adult worms and inhibits phosphorylase activation, resulting in decreased glycogen levels in the schistosomes (43). Mahmoud and Warren (54) report remarkable suppression of cell-mediated immunity by niridazole resulting in reduced schistosome egg granuloma and delayed footpad swelling to

soluble egg antigens. In addition, niridazole is more effective in maintaining allograft survival to strong histoincompatibility antigens than other immunosuppressive agents currently used in organ transplantation (55). Furthermore, niridazole apparently lacks the toxic side effects of other immunosuppressants. Decrease in white blood cell counts, bone marrow, or increased susceptibility to infection are not noted (41,86). The mechanism of action of niridazole is not understood but may involve suppression of non-specific inflammatory reactions (72). Influence of this drug on humoral immunity has not been extensively studied, but a recent report indicates that continued niridazole therapy depresses antibody production (67).

It is noted earlier that immunosuppression may affect one or more phases of the immune response, from antigen processing, through cell differentiation and proliferation, and eventually antibody secretion and actions of sensitized cells. Glucocorticosteroid hormones are thought to have the unique attribute of affecting each stage of the immune response. These are the most commonly used drugs for the suppression of undesirable immune or inflammatory responses, especially with regard to homotransplantation. Even though clinical application is extensive, considerably less is known about the cellular responses through which the effects occur. When interpreting data with corticosteroids, species differences must be kept in mind. Mouse, rat, hamster, and rabbit appear to be steroid-sensitive while man, guinea pig, and monkey are relatively steroid-resistant (23). The exact basis for these differences is unknown.

One of the effects of corticoids is upon peripheral leukocyte populations. Transient but selective decreases in circulating lymphocytes and monocytes and an increase in neutrophils occur in man within four to six hours following corticosteroid therapy but these cells return to normal levels within 24 hours (36, 71). Circulating T lymphocytes decrease, probably as a result of redistribution to other areas of the body (26,34,35,108). During the period of lymphocytopenia functionally mature lymphocyte subpopulations are detected in the bone marrow, even though total numbers of bone marrow lymphocytes remain constant. Therefore, the bone marrow assumes increased immunocompetence while the circulating lymphocyte population becomes immunosuppressed.

Corticosteroids also have an influence on the humoral immune response. Corticosteroids are used to control some of the symptoms associated with IgG and IgM humoral antibody responses in certain allergic and autoimmune diseases. It is reasonable to ask whether corticoids prevent induction and/or expression of the immune response and what cell types are affected. Administration of methylprednisolone to human volunteers results in decreased IgG and IgA but no significant decrease in IgM levels (17). It was shown by studies with ^{125}I-IgG that corticosteroid-induced decreased IgG synthesis accounts for 90% of the decrease in serum IgG levels. Increased catabolism of IgG accounts for the remaining 10%. Cohen (25) repopulated lethally irradiated mice with bone marrow and either normal or hydrocortisone-treated thymus cells. Mice in both groups mounted equal antibody responses to SRBC's, which is surprising since thymus glands of cortisone-

treated mice contain only 5% of the lymphocytes normally present. This suggests that 5% of the thymus lymphocytes which are corticosteroid-resistant are also immunocompetent. Antibody production by spleen cells, a function of T and B cells, is suppressed but may be restored by administration of bone marrow cells of normal mice. The B cell precursor thus appears to be the corticosteroid-sensitive step in induction of humoral immunity. Cohen finds mature antibody secreting cells to be corticosteroid-resistant. In contrast, Butler (17) reports that human volunteers on methylprednisolone therapy develop high levels of circulating antibody to keyhole limpet hemocyanin, diphtheria toxoid, tetanus toxoid, adenovirus vaccine, and influenza-virus vaccine, even though total IgG serum concentration decreases. He also notes that naturally acquired herpesvirus titers drop, indicating inhibition of ongoing antibody synthesis or expression of humoral immunity.

Corticosteroids may also alter cell-mediated immunity such as delayed hypersensitivity, contact sensitivity, allograft rejection, and graft versus host (GVH) reaction. These phenomena are regulated by T lymphocytes which contact specific antigen and are stimulated to divide and secrete soluble factors. The visible inflammatory lesion is a result of localization of nonspecific cellular elements. Corticosteroids inhibit the inflammatory response, but is it at the level of the lymphoid initiator cell or the ensuing nonspecific response? In order to discuss the differential sensitivity of T cells, it is necessary to consider each of the T Cell functions. It has already been pointed out that

all but 5% of the thymus lymphocytes are corticosteroid-sensitive, but the remaining cells manifest immunocompetence in eliciting GVH and helper cell function in lethally irradiated hosts. Cohen (25) establishes that hydrocortisone inhibits the GVH reaction. He transfers parental strain lymphoid cells, control and hydrocortisone-treated, to F_1-hybrid hosts and notes enlarged spleens in only animals receiving control lymphoid cells. Parental lymphocytes apparently home to the recipient's spleen and are stimulated to divide by histoincompatibility antigens. These stimulated donor cells release factors which result in proliferation of the recipient's cells and also in a nonspecific inflammatory response to injury. Spleen and thymus cells of hydrocortisone-treated parental donors, however, do not protect F_1-hybrids from the GVH reaction. Therefore, he postulates that hydrocortisone suppresses the nonspecific inflammatory response and not the T cells initiating the GVH reaction. In contrast to the steroid resistance of T cells activated by antigen, T cells activated by the nonspecific mitogen phytohemagglutinin (PHA) are corticosteroid-sensitive (13,19,36). After activation by antigen or mitogen, T lymphocytes affect other cells via direct cell contact or through the production of soluble mediators. Little is known about the <u>in vivo</u> effects of corticosteroids on lymphocyte-produced mediators of cellular immunity. According to Williams and Granger (105) corticoids inhibit PHA-induced lymphotoxin production by human lymphocytes. Furthermore, macrophage migration inhibitory factor (MIF) is released by lymphocytes of corticosteroid-treated guinea pigs, but macrophages of these animals are unable to respond to MIF. Thus, steroid hormones

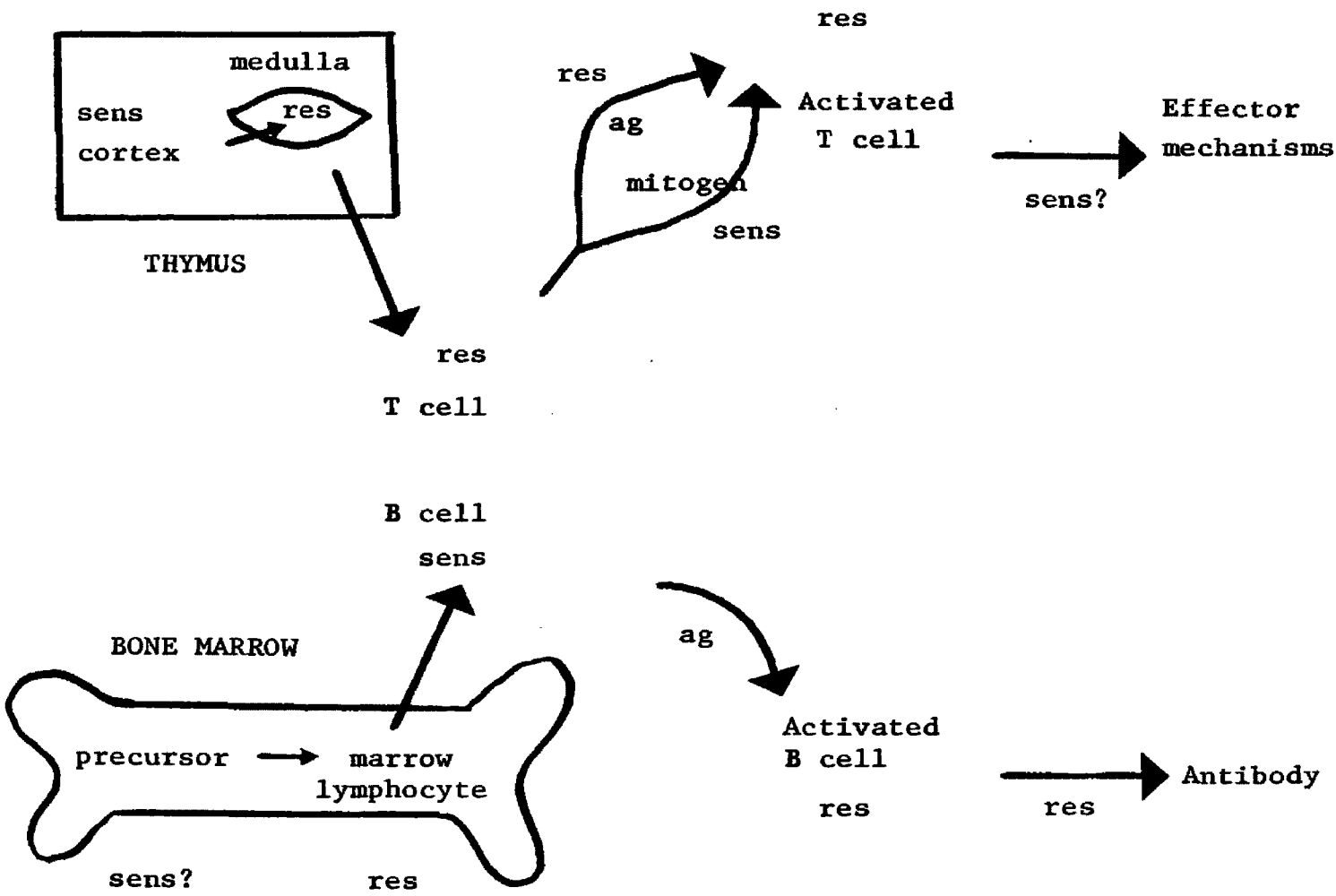
appear to interfere with the effector limb of lymphocyte mediator function. In summary, figure 1 shows the differential sensitivity of lymphoid subpopulations to corticoids.

Considerable work has been done to elucidate the mechanism of glucocorticoid action. Recent work suggests that physiological responses to steroid hormones are mediated through specific intracellular protein receptors (10). Steroids penetrate the membrane and bind to the receptors inside the cell. Loss of receptor correlates with loss of steroid sensitivity. Steroid binds with the receptor, which probably undergoes a conformational change allowing close association of the receptor-steroid complex with DNA. Accumulation of mRNA then occurs, but the mechanism does not explain the exertion of an inhibitory effect by glucocorticoids during increased RNA synthesis.

Whole body irradiation by X-irradiation is one of the earliest forms of immunosuppression. Inhibition of antibody response and suppression of graft rejection can be accomplished by administration of X-ray prior to injection with antigen. The treatment destroys lymphocytes and stem cells but also damages severely other hematopoietic and tissue cells. Local application of irradiation to tumors or grafted skin or organs reduces the toxicity problem but also limits the contribution of X-irradiation as an immunosuppressant (51).

A variety of biological agents and surgical treatments are also available for suppression of the immune response. Anti-lymphocyte serum is a potent inhibitor of cellular immunity and

Figure 1. Fluctuation of sensitivity and resistance of lymphoid cells to cortisone (23). res: resistant to cortisone
sens: sensitive to cortisone
ag: antigen



does not produce the toxic side effects of other immunosuppressive agents (75). Antilymphocyte serum binds to the recirculating population of antigen-reactive cells, which are then eliminated by cytotoxic destruction. Humoral immune responses to T-cell-dependent antigens may also be decreased by antilymphocyte serum. Specific elimination of cellular immunity by this antiserum increases the possibility of lowered host resistance and generation of spontaneous neoplasms. Viral infections with non-oncogenic (68) and oncogenic (74) viruses may cause immunosuppression of either compartment of the immune response. Surgical removal of thymus, spleen, or lymph nodes may result in depletion of specific populations of cells.

The opportunities for immunosuppression, table 2, range from specific to nonspecific, physical, chemical, and biological agents. Variations in dosage, schedule, and route of administration may result in suppression of virtually every phase of cellular and/or humoral immunity. However, the immune responses most affected by the nonspecific immunosuppressive agents are listed in table 3.

Statement Of The Problem

Four drugs are used in this study: hydrocortisone acetate, azathioprine (Imuran), niridazole (Ambilhar), and amethopterin (Methotrexate). These drugs are classified as corticosteroid, purine antimetabolite, cytotoxic chemical, and folic acid antagonist respectively. Immunosuppressive drugs are used extensively although their effects on the immune system are poorly understood. For this reason, this study was designed to investigate the temporal response of a variety of immunosuppressive drugs on the

Table 2. Immunosuppressive agents (nonspecific).

Physical agents	Irradiation
Chemical agents	Alkylating agents Purine analogs Pyrimidine analogs Folic acid antagonists Antibiotics Plant alkaloids Carcinogens
Biological agents	Enzymes Hormones Antilymphocyte serum Antimacrophage serum
Living organisms	Viruses Parasites
Removal of lymphoid tissue	Thymectomy Splenectomy Bursectomy in birds

Table 3. Compartments of the immune response most affected by various immunosuppressive agents.

Agent	Compartment
A) Cytotoxic chemicals	Humoral
alkylating agents	
antimetabolites	
folic acid antagonists	
antibiotics	
plant alkaloids	
B) Corticosteroids	Cell-mediated
C) Irradiation	Humoral
D) Biologic agents	
thymectomy	Cell-mediated
antilymphocyte serum	Cell-mediated
enzymes	Cell-mediated
bursectomy	Humoral

T and B lymphocyte compartments of the immune response using the mouse system as a model. Understanding mechanisms of action may provide a rational basis for immunosuppressive therapy.

Chapter 2

MATERIALS and METHODS

Animals

Male Swiss-Webster mice were obtained from a colony maintained by Rocky Mountain Laboratory, Hamilton, Montana. Mice used for all investigations were five to six weeks of age.

Drugs

Hydrocortisone acetate (Microfine), Towne, Paulsen, and Co., Inc. Monrovia, California 91016, was suspended in cortisone diluent (Table 4) to concentrations of 10 mg/ml, 20 mg/ml, or 40 mg/ml. Mice were injected subcutaneously in the nuchal area with 150 mg/kg, 350 mg/kg, or 600 mg/kg body weight.

Imuran^{R} brand azathioprine, Burroughs Wellcome Co., Research Triangle Park, North Carolina 27709, was dissolved in 0.85% sterile saline to a concentration of 10 mg/ml. Mice were injected subcutaneously in the nuchal area with 150 mg/kg body weight.

Miridazole, Ciba Pharmaceutical Co., Division Ciba-Geigy Corp., Summit, New Jersey 07901, was suspended in sterile distilled water to concentrations of 2 mg/ml and 20 mg/ml. The drug was administered orally by using a 1 ml syringe with attached beveled and bent (45° angle) 16 gauge needle which could be inserted deep into the animals throat.

Methotrexate, Lederle Labs, Division, American Cyanamid Co., Pearl River, New York 10965, was dissolved in 0.5 M sterile Na_2CO_3 to a concentration of 40 mg/ml. Mice were injected intraperitoneally

with 250 mg/kg body weight.

All drugs were administered 10, 8, 6, 4, 2 or 1 day prior to antigen or on the day antigen was administered.

Humoral Immune Response by Hemagglutination

Rabbit blood was obtained from an adult New Zealand white rabbit by cardiac puncture. Equal volumes of Alsevers solution (Table 5) and blood were mixed and allowed to equilibrate for 48 hours. Cells were washed three times with 0.85% saline and centrifuged at 1500 rpm on a Universal centrifuge Model V for five minutes each time. Red blood cells were resuspended in 0.85% saline to a concentration of 2.5%. Mice were immunized intravenously via the tail vein with 0.2 ml of the 2.5% rabbit red blood cell suspension using five mice/group. Mice were bled from the axillary artery five days later, and serum was collected. All sera were heat inactivated at 56°C for 30 minutes. Hemagglutination was performed on serial two-fold dilutions of serum by the microtiter method with the materials of Cooke Engineering Co., Alexandria, Virginia. Rabbit red blood cells were washed three times with 0.85% saline and resuspended to a concentration of 2% in 1% normal rabbit serum, which was prepared in saline. Diluent was 1% normal rabbit serum in saline. The reaction was incubated at 37°C for one hour then overnight at room temperature.

Delayed Hypersensitivity (DH) Response by Oxazolone Sensitization

According to the method of Asherson and Ptak (2), seven mice/group were sensitized prior to or following drug administration by applying 3% oxazolone (4-Ethoxymethylene-2-phenyl oxazolone) BDH Laboratory Reagents, BDH Chemicals Ltd., Poole, England, in

absolute ethanol to the preshaved abdomen using 0.1 ml/mouse. The solution was rubbed in with the lengthwise edge of a 20 guage needle. Seven days following the final day of drug treatment or oxazolone sensitization, 2% oxazolone in absolute ethanol: olive oil (1:1) was applied to both ears of sensitized mice, 1 drop/ear delivered from a 25 gauge needle. The emulsion was rubbed in extensively with a cotton tipped applicator stick. Skin tests were read at 24 hours by measuring double ear thickness with a Schnelltaster. Results were reported as group averages and percent of control ear thickness.

Friend Disease Virus Infection of Drug Treated Animals

Friend Disease Virus (FDV) was obtained from the Stella Duncan Research Institute, University of Montana, Missoula, Montana. The institute prepared stock pools of the virus after three passages were made in Swiss-Webster mice at The Rocky Mountain Laboratory, Hamilton, Montana.

To prepare working stock suspension, 30 male mice were injected with 0.2 ml of $10^{-2.0}$ dilution of FDV. After 28 days the mice were sacrificed and the spleens were removed and suspended in cold medium, consisting of chemically defined Medium 199 (m-199), GIBCO, Grand Island, New York, and diluted to a concentration of 20%. After teasing the tissues over a wire mesh and placing into two 40 ml plastic centrifuge tubes, the suspension was centrifuged at 1000 rpm in an RC-2 Sorvall refrigerated centrifuge for 15 minutes at 4°C. The supernatant was then collected and centrifuged at 6000 rpm for 30 minutes at 4°C. The top 2/3 to 3/4 of the clear

supernate was removed and pooled. Aliguots of 0.6 ml were distributed in 2 ml ampoules, sealed, and frozen in a Revco freezer at -70°C.

Following drug treatment, mice were injected intravenously with 0.2 ml of a $10^{-2.3}$ dilution of FDV using 10 mice/group (96). On day 14 all animals were weighed and autopsied. Spleens were removed, weighed, and fixed in Bouin's fixative (Table 6). Viral plaques were counted after 18 hours fixation, according to Axelrad and Steeves (3).

Blood Cell Counts and Differentials

Blood was collected from the axillary artery of drug treated mice. Whole blood was diluted 1:500 with 0.85% saline for red blood counts and 1:20 with Turk's diluting fluid (Table 7) for leukocyte counts. Counting was accomplished using a Neubauer hemocytometer. Differential leukocyte counts were performed on blood smears stained with Giemsa (Table 9).

Immunofluorescence

Drug-treated mice were sacrificed and their spleens immediately placed in cold m-199 plus 10% Fetal Calf Serum and 10% triple distilled water containing five units preservative free heparin/ml. Spleens were teased through a wire mesh into 10 ml cold media. The cell pellet was resuspended in 10 ml cold media and layered over 10 ml of Ficoll-Hypaque (Table 8) in 40 ml Sorvall centrifuge tubes. Centrifugation was carried out at 1500 rpm for 20 minutes at 4°C in an International portable refrigerated centrifuge model PR-2. The white lymphocyte layer was removed and washed three times in

cold media. Lymphocytes were counted using a Neubauer hemocytometer and were resuspended in media to a concentration of 5×10^6 cells/ml. Cells (0.1 ml) were placed in Corning centrifuge tubes (2 mm X 30 mm) and 0.1 ml of a 1:4 dilution of mouse anti-serum globulin (rabbit origin) conjugated with fluorescein isothiocyanate (FITC), Colorado Serum Co., 4950 York Street, Denver, Colorado, was added. All tubes were incubated for 45 minutes at 4°C. Following incubation cells were centrifuged at 1200 rpm for one minute and washed three times with 0.4 ml cold media. Lymphocytes were resuspended in two drops of media and placed on a glass slide with cover slip. Paraffin was used to seal the edges of the cover slip. All slides were viewed using a Zeiss fluorescence microscope with excitation filters I and III and barrier filters 50 and 0. A total of 200 cells/slide were counted, fluorescent cells being scored as B cells and non-fluorescent cells as T cells. Cells showing diffuse fluorescence rather than strictly membrane fluorescence were considered nonviable and were not counted.

Table 4. Cortisone diluent.

Saline 0.85%	98.7 ml	
Tween 80	0.4 ml	Nutritional Biochemical Corp.
Benzyl Alcohol	0.9 ml	J.T. Baker Chemical Co.
Carboxy methyl cellulose	0.5 g	Svenka Cellulosa AB Sundsvall, Sweden

Add in order given.

Table 5. Alsevers solution.

Dextrose	20.5 g
Sodium citrate (dihydrate)	8.0 g
Citric acid (monohydrate)	0.55 g
Distilled water	to 1 liter

Dissolve ingredients successively in distilled water. Autoclave at 15 psi for 15 minutes. pH should be 6.1. Use 1 part blood: 1 part Alsevers.

Table 6. Bouin's fixative.

Picric acid (saturated aqueous, filtered)	75 ml
Neutral formalin (filtered) 20 g sodium acetate plus 100 ml 40% formalin	25 ml
Glacial acetic acid	5 ml

Table 7. Turk's white blood cell diluting fluid.

Glacial acetic acid	2 ml
Aqueous gentian violet 1% w/v	1 ml
Distilled water	100 ml

Mix and filter.

Table 8. Ficoll - Hypaque.

1. Dissolve 9.34 g sodium diatrizoate, Winthrop Laboratories, 90 Park Ave., New York, New York 10016, in 30 ml distilled water and adjust to pH 7.3.
2. Add 72 ml distilled water.
3. Dissolve 6.28 g Ficoll, Pharmacia Fine Chemicals AB, Uppsala, Sweden, in above solution.
4. Encase bottle in foil and autoclave for 15 minutes at 121°C.

Table 9. Giemsa stain for mouse blood cells.

Stock giemsa:

1. Dissolve 0.5 g giemsa powder in 33 ml glycerine.
2. Heat at 55 - 60°C for 1½ to 2 hours.
3. Add 33 ml absolute methanol.

0.15 M phosphate buffered saline pH 6.4: (PBS)

0.85% saline	100 ml
0.15 M Na_2HPO_4	32.2 ml
0.15 M KH_2PO_4	67.8 ml

1. Fix smears in absolute methanol for 10 seconds or longer.
2. Allow slides to air dry.
3. Rinse a small test tube with 5 ml distilled water and add another 5 ml distilled water to the tube. Add a few crystals of hematoxylin to the test tube. If the water appears yellow the pH must be adjusted. Add about 0.1 ml 1% KCO_3/ 200 ml distilled water. The color of the water in the 5 ml tube should be light pink in color when hematoxylin is added.
4. Use 0.2 ml stock giemsa/ml PBS pH 6.4. Flood smear with 3 ml/slide for 30 seconds.
5. Float off stain with adjusted distilled water.

Chapter 3

RESULTS

Hydrocortisone Acetate

The initial experiments were designed to investigate the temporal effects of cortisone on humoral and cell-mediated immunity. In general cortisone markedly diminished both components of the immune response for four days following drug administration. Administration of antigen shortly after administration of drug resulted in a decrease of circulating antibodies to rabbit red blood cells (Figure 2). Increase in time intervals between drug treatment and antigen administration effected a recovery of the antibody response which was apparent by day 10. There were no significant variations in the antibody titer of groups of mice receiving different doses of hydrocortisone acetate. Since red blood cells are T-lymphocyte-dependent antigens, it was not possible to determine whether suppression occurred at the level of the T-cell precursor, B-cell presursor, or both. Therefore, the experiment was repeated using the T-cell-independent antigen lipopolysaccharide. With this antigen, a decreased antibody titer was noted only one or two days following cortisone treatment.

The ability of mice sensitized to oxazolone and treated with cortisone to express a delayed reaction was studied. This ability to express a DH response was essentially abolished during the first four days following cortisone treatment but gradually increased to

control levels by day 10 (Figure 3). While the pattern of reaction was similar in mice given different doses of cortisone, recovery was much less in those given larger doses of drug. However, if mice were given cortisone and then sensitized with oxazolone in order to determine the action of drug on the afferent arm, the initial lack of DH at day zero was followed by gradual increase in skin sensitivity culminating in marked enhancement of the DH at day 4, after which the response declined and approached control levels (Figure 4). Skin tests were repeated on the oxazolone pre-sensitized mice between ten and twenty days following cortisone treatment and from 30 to 60% recovery of the DH response was noted. The nonspecific inflammatory response was eliminated as shown by the absence of inflammation in ears of skin-tested hydrocortisone-treated mice. Both the affector and effector arms of the DH reaction were disturbed in mice treated with cortisone but to different degrees at various times after administration of the drug.

The weight of the spleens of drug-treated mice showed a remarkable decrease through day 4 and was followed by a gradual increase which was less apparent in mice given the larger doses of cortisone (Figure 5). The weight of spleens of control mice, however, always increased in size with age.

Interestingly, cortisone-treated mice (150 mg/kg) were as susceptible to FDV infection as control mice (Figure 6). Other investigators (21,76) have noted that mice with suppressed humoral immunity do not exhibit increased spleen weights or spleen viral

foci (plaques) which are quantitative measurements used to detect FDV infection. This may suggest that hydrocortisone immunosuppression is directed against the T-lymphocyte precursor rather than the B-lymphocyte precursor. Data from the 350 mg/kg cortisone-treated mice appeared confusing. Viral foci were nearly confluent in spleens of all infected mice, but spleen weights fluctuated considerably. Spleen sizes of infected mice increased over control spleens if FDV was given one or two days following cortisone treatment, decreased rapidly when FDV was administered four days following drug treatment, and gradually increased at time intervals thereafter. Generally, spleen size and plaque number correlate well, whereas in this experiment spleen weight was low even while spleen plaque numbers were high.

Spleen B cell populations were assayed by immunofluorescence. Data was difficult to obtain during the first days immediately following drug administration because of the drastic reduction in spleen size. However, on the basis of other sampling intervals total spleen B cell populations appeared only minimally depressed throughout the course of treatment (Figure 7).

In order to determine the effect of different doses of cortisone on populations of peripheral white blood cells, total white and red blood cell counts and differentials were performed. Variations for total red blood cell counts were within normal ranges in all cases. Total white blood cell counts of mice treated with 150 mg/kg cortisone fluctuated around the lower range of counts for untreated controls. Mice treated with 350 mg/kg cortisone had counts lower than normal for the first two days, peaked

sharply on day 6, and then gradually declined. Leukocyte responses of mice treated with high doses of cortisone (600 mg/kg) closely resembled those of mice treated with the intermediate dose. Relative to the total white blood cell count, neutrophil numbers were elevated through the fourth or sixth day in all cortisone-treated groups and then started a gradual decline (Figure 8). Lymphocyte numbers dropped through day 6 and then began a gradual recovery. Monocyte levels remained depressed throughout cortisone treatment. These selective alterations in leukocyte populations agree with data obtained from humans on corticosteroid therapy (36).

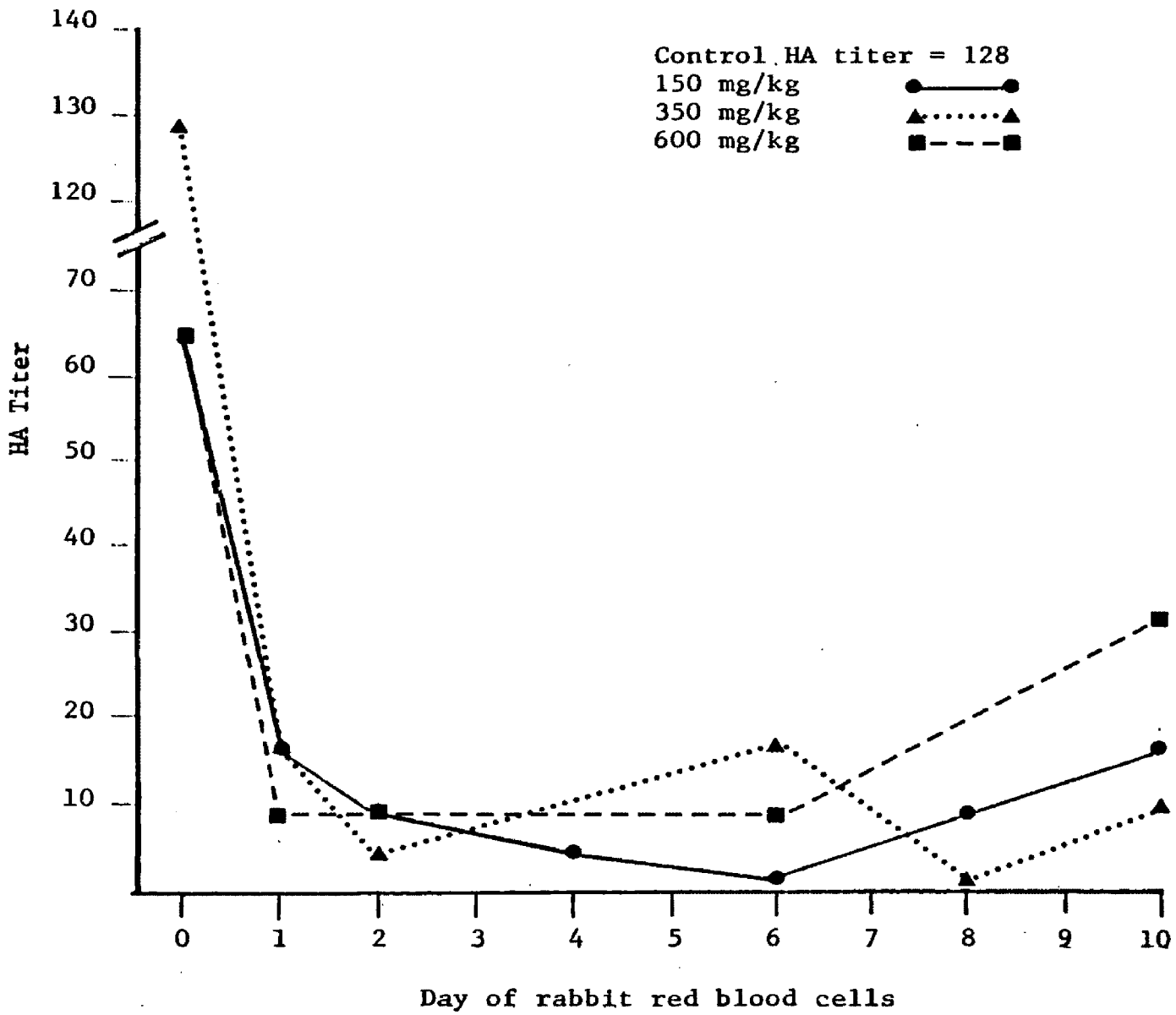


Figure 2. Effect of hydrocortisone acetate (administered on day zero) on the humoral immune response of mice to rabbit red blood cells. Each point represents pooled sera of five mice. HA = hemagglutination titer.

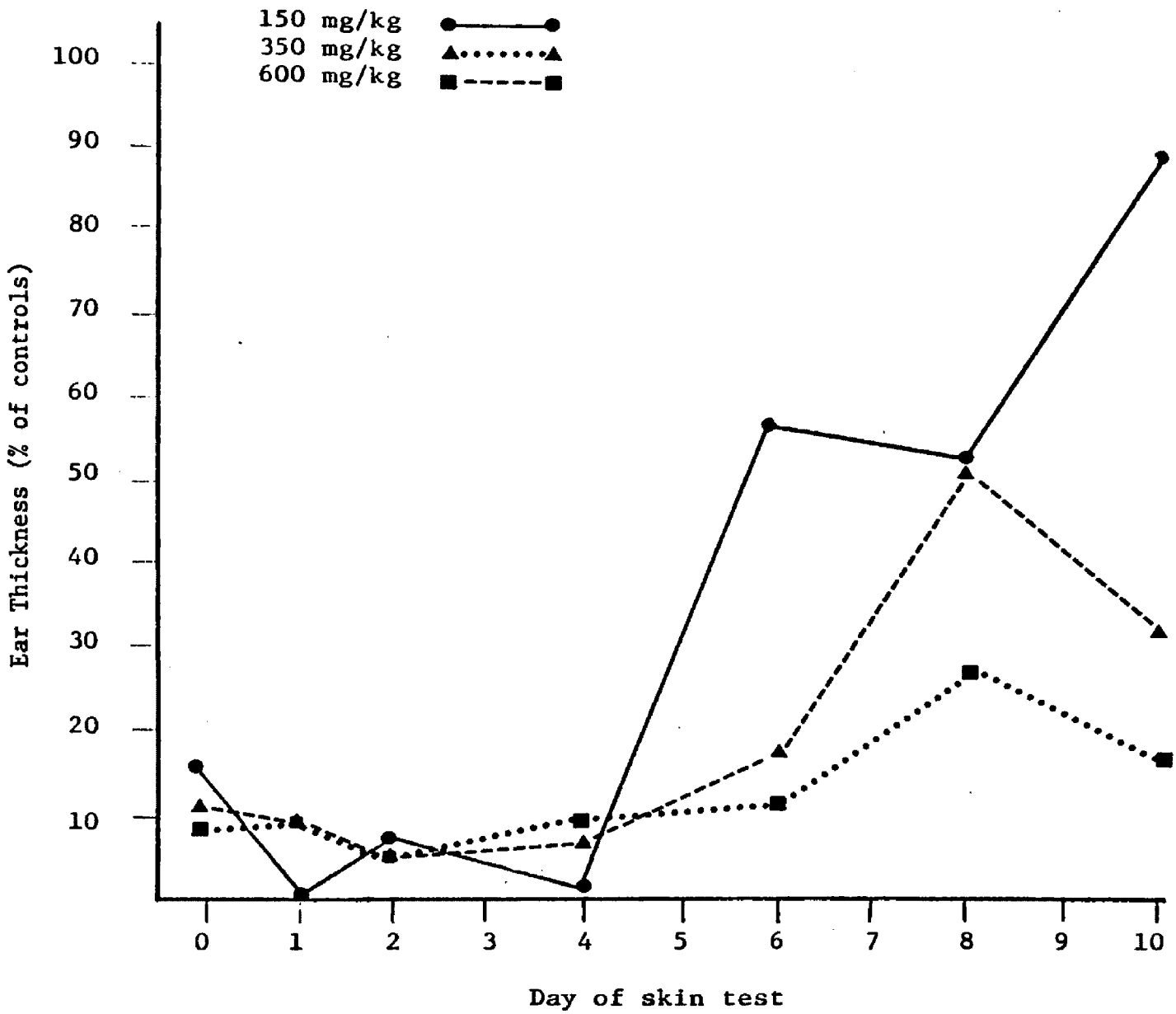
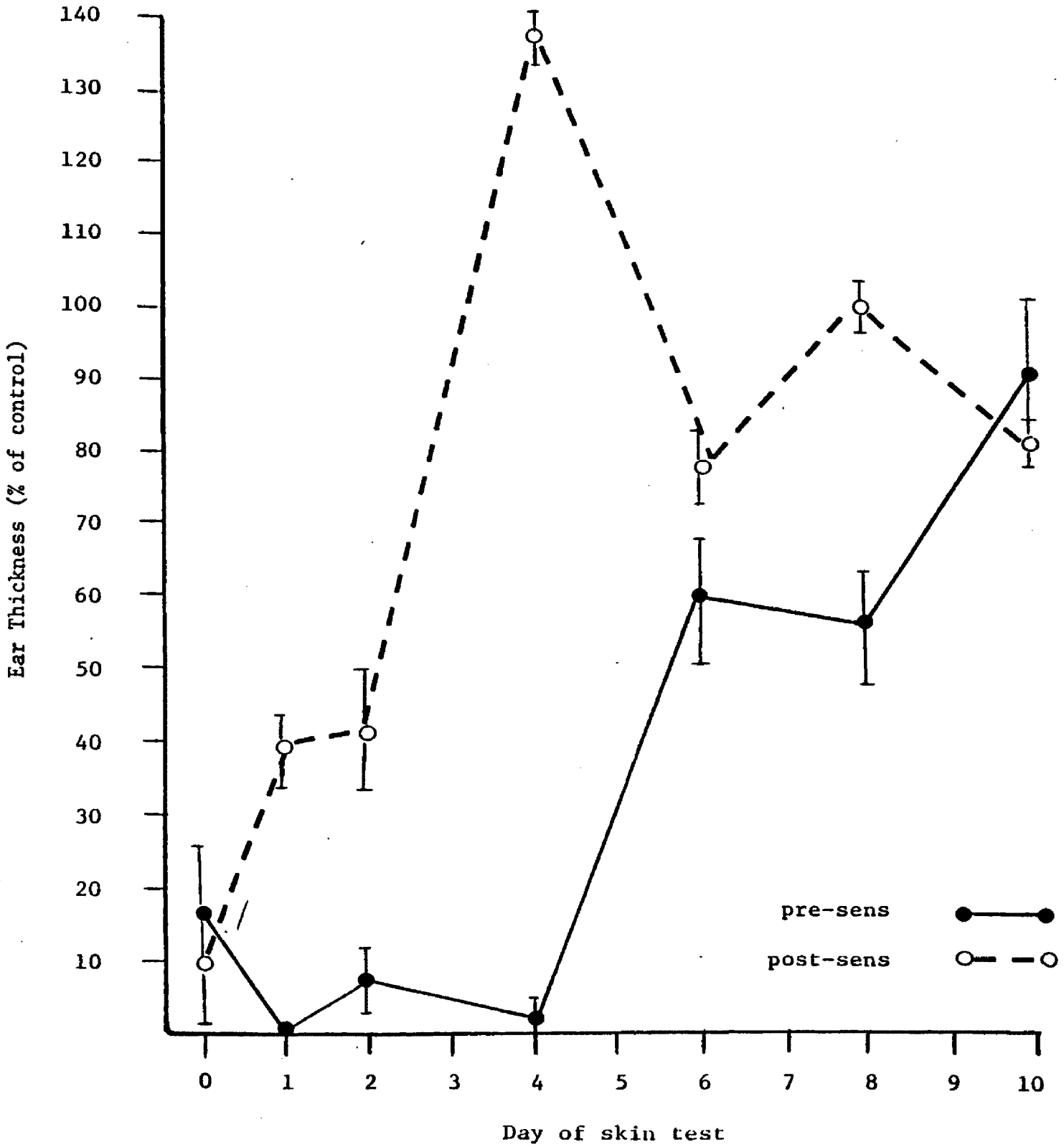


Figure 3. Effect of hydrocortisone acetate (administered on day zero) on the DH response of mice previously sensitized with oxazolone. Each point represents pooled measurements from both ears of seven mice.



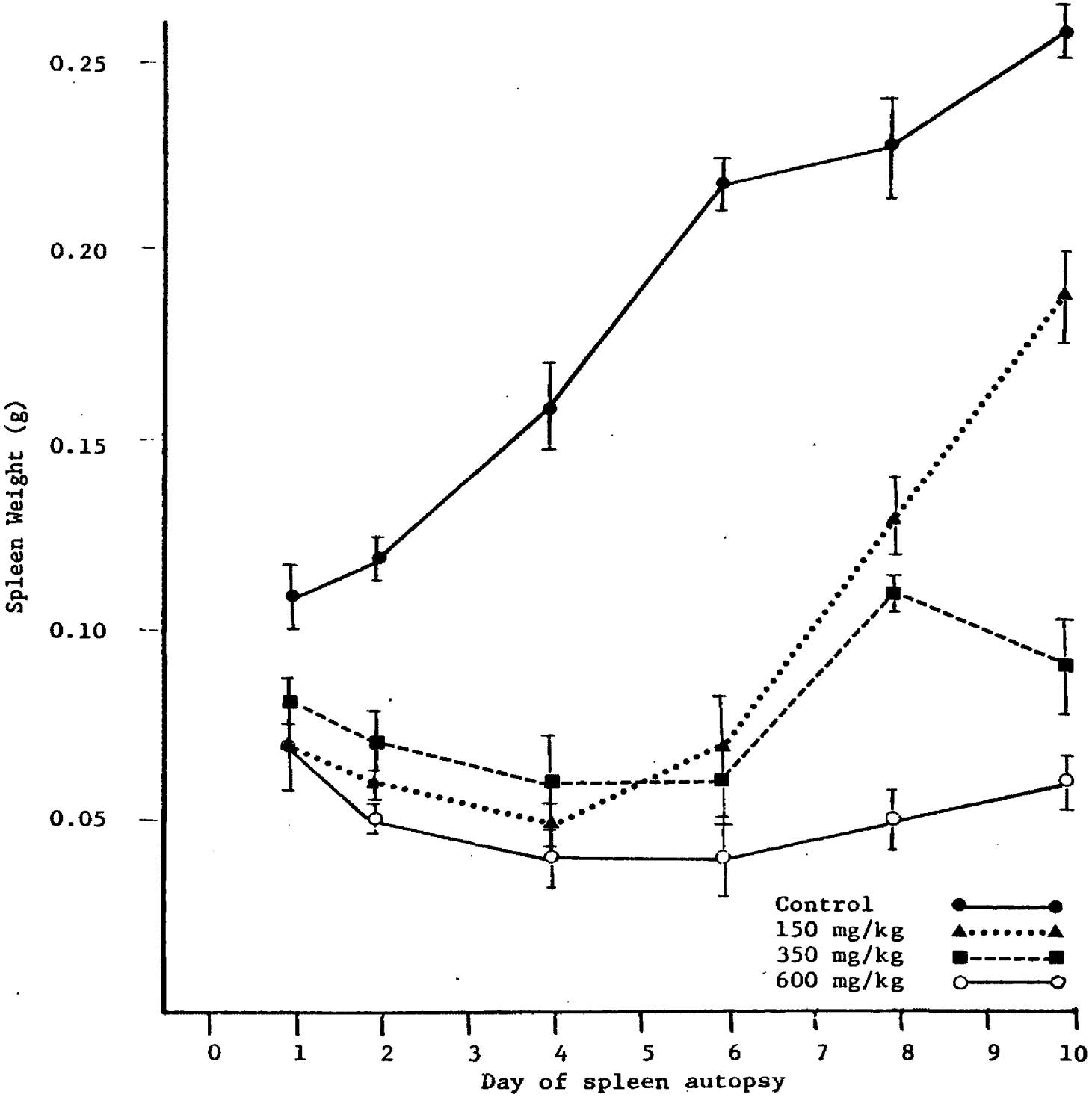


Figure 5. Effect of hydrocortisone acetate (administered on day zero) on spleen weights of treated mice.

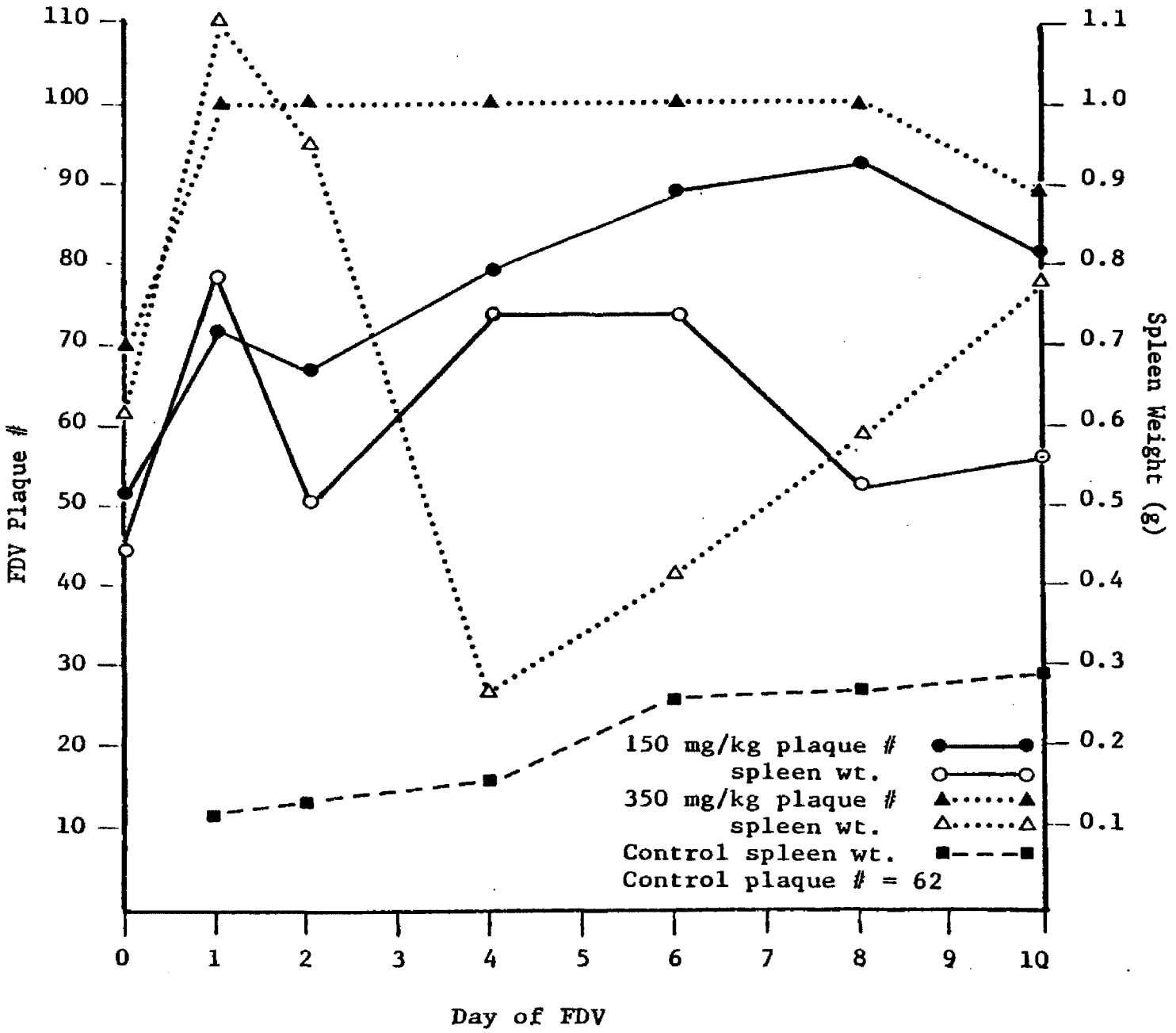


Figure 6. Effect of hydrocortisone acetate (administered on day zero) on susceptibility of mice to FDV infection. Each point represents the mean of the results from ten mice.

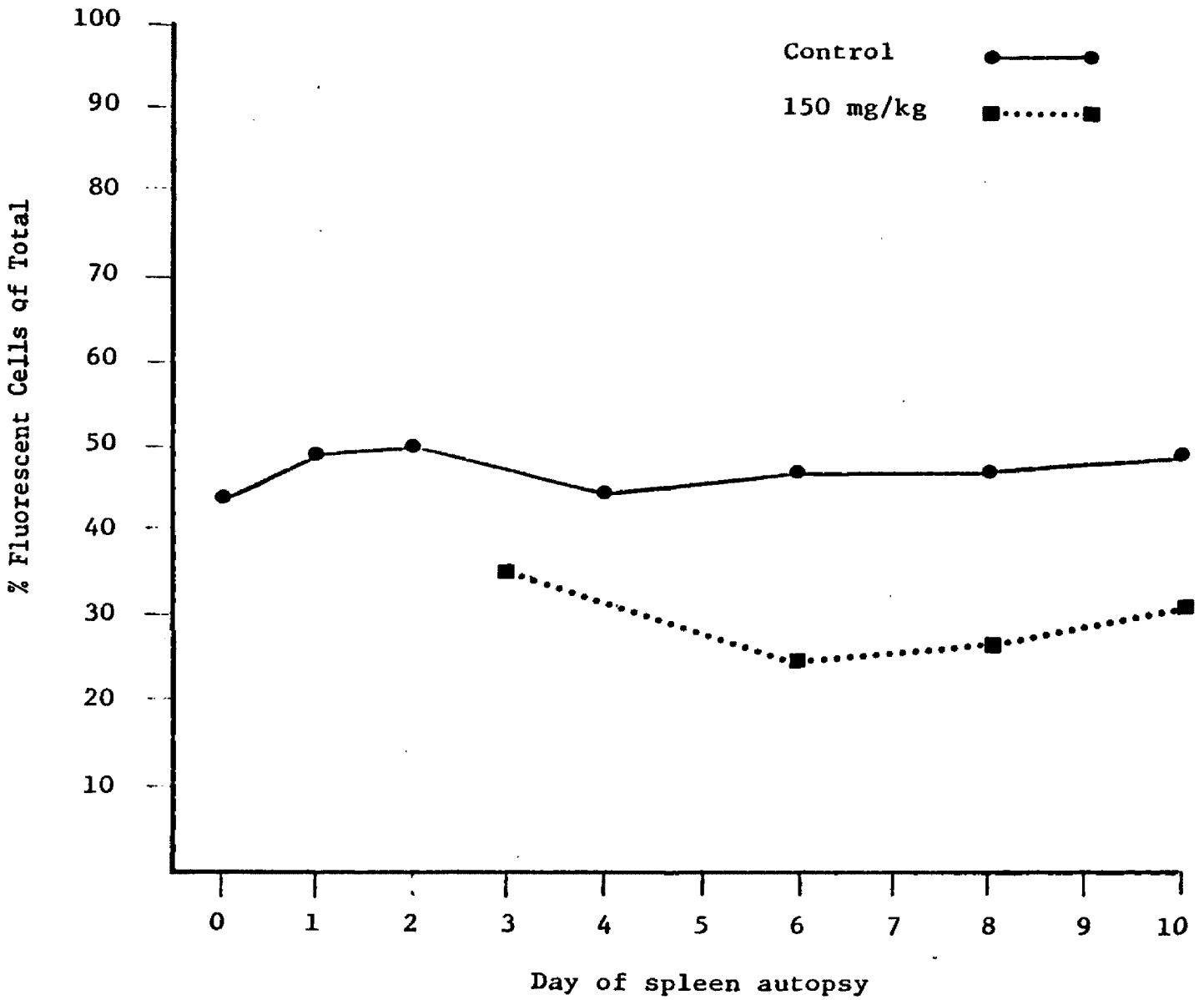


Figure 7. Effect of hydrocortisone acetate (150 mg/kg administered on day zero) on B cell populations in spleens of mice as assayed by fluorescent antibody.

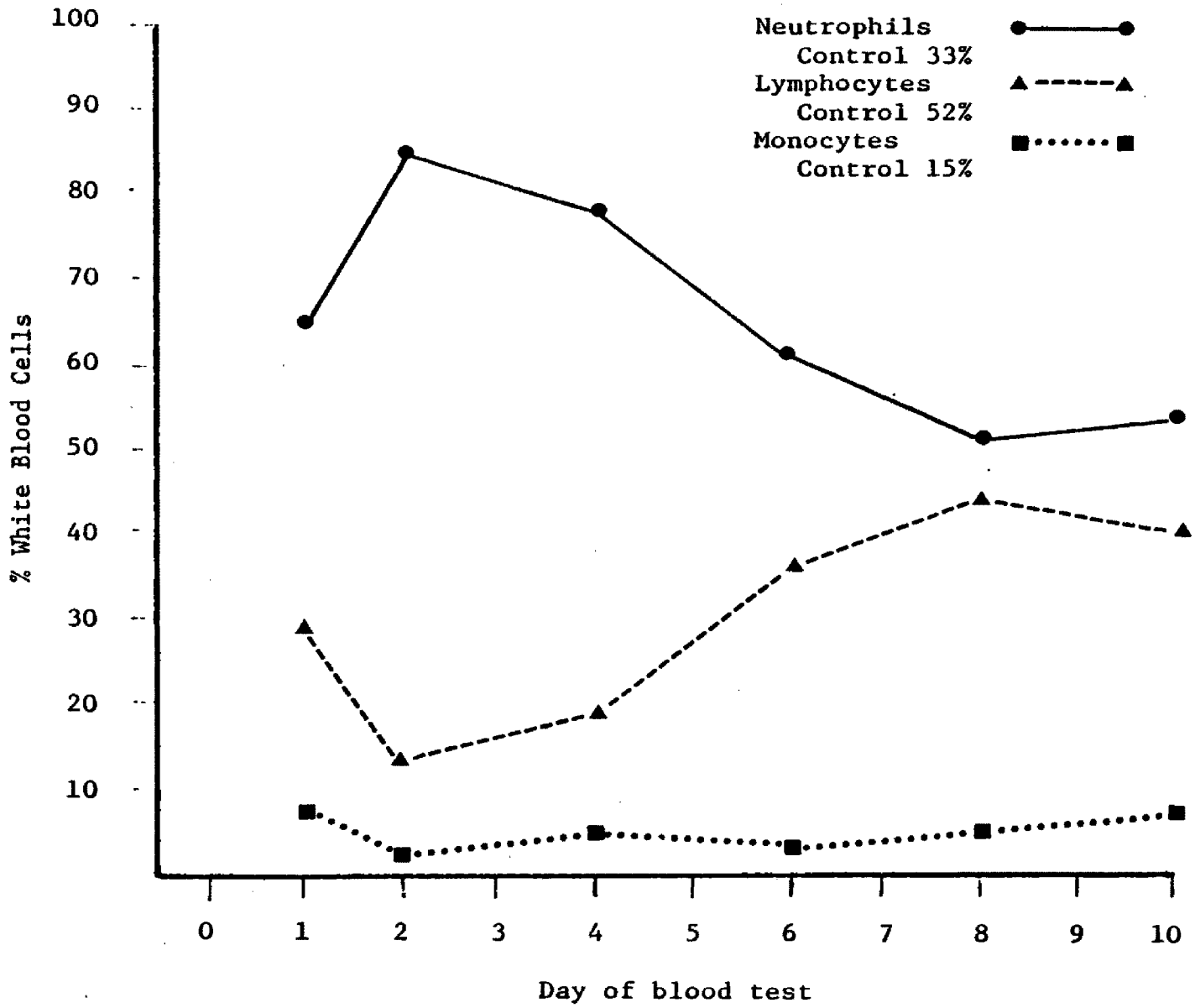


Figure 8. Effect of hydrocortisone acetate (150 mg/kg administered on day zero) on populations of circulating leukocytes.

Azathioprine

Based on experiments conducted in this study, immunosuppression by azathioprine was not particularly impressive. Humoral antibody responses as measured by hemagglutination of rabbit red blood cells were depressed by only one 2-fold dilution when drug was given either on the day of rabbit red blood cells or one day prior to antigen (Figure 9). A sharp peak occurred when drug was given eight days prior to antigen. These results were in agreement with those of Tripathy (98) who found that production of 19S antibody to Listeria monocytogenes was not suppressed and, in fact, appeared to be enhanced.

There was a heightened DH response to oxazolone on the eighth day following drug treatment in mice previously sensitized with this agent (Figure 10). The other deviation observed was depression of the DH response between four and six days following administration of the drug. Since azathioprine interferes primarily with induction of immunity, lymphocytes sensitized to oxazolone prior to drug treatment were present and functional several days following challenge with azathioprine. Depression of DH at day 4 may indicate that azathioprine eliminates some of these oxazolone-sensitized lymphocytes. Apparently compensation by production of more sensitized cells is manifested by day 8 following drug treatment.

Susceptibility of azathioprine-suppressed mice to FDV was decreased when FDV was given within two days following drug treatment but compared closely with control animals thereafter (Figure 11).

Apparently B cells are important for viral replication and intervals of suppression of humoral immunity correlated with decreased spleen weight and plaque numbers during FDV infection.

Spleen weights of drug-treated mice revealed no variation from control spleen weights except for a sharp peak in weight at day 6 (Figure 12). This rise may correlate with B cell increase during peak antibody titer and depressed DH at this time.

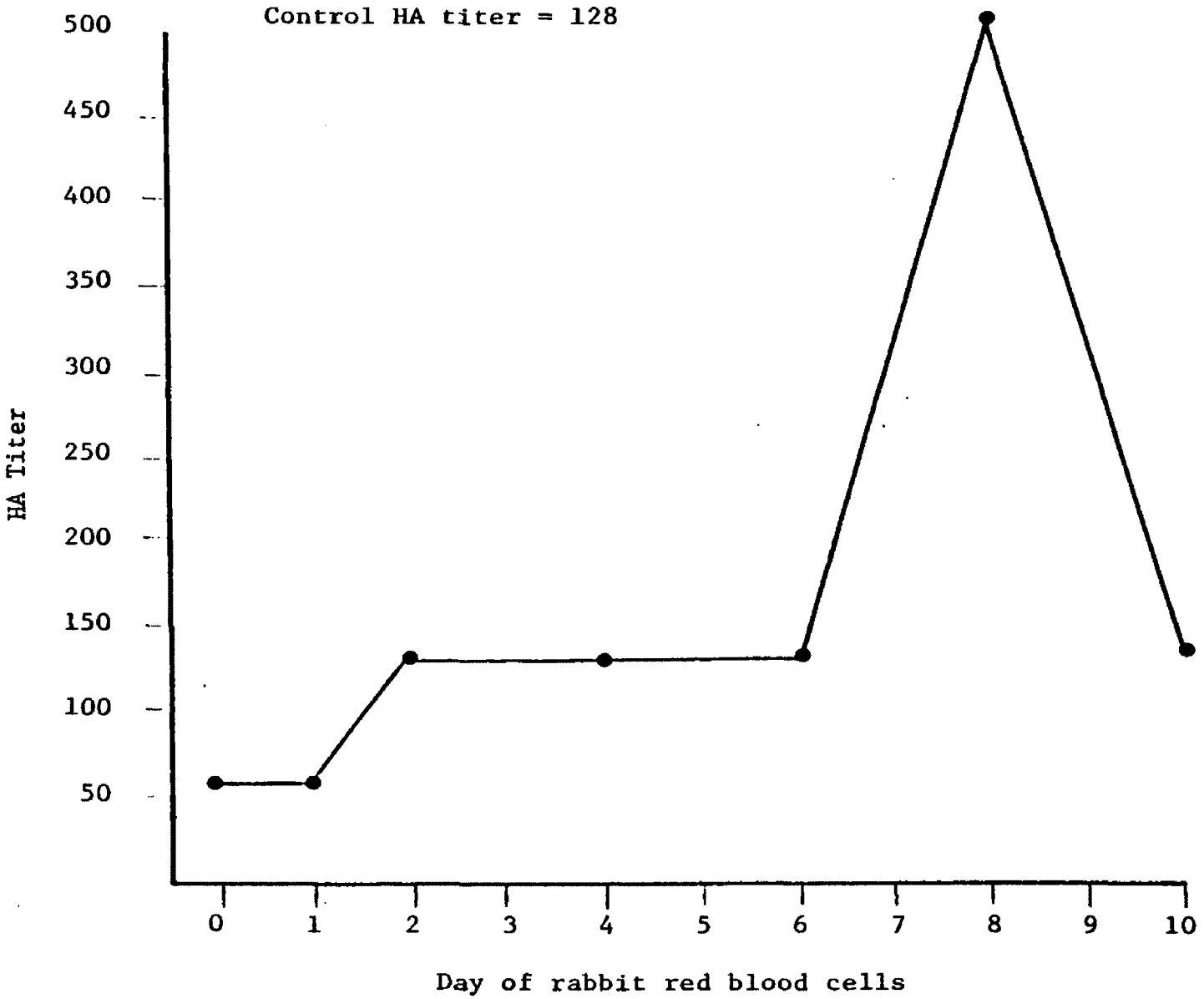


Figure 9. Effect of azathioprine (150 mg/kg administered on day zero) on the humoral immune response of mice to rabbit red blood cells. Each point represents pooled sera of five mice. HA = hemagglutination titer.

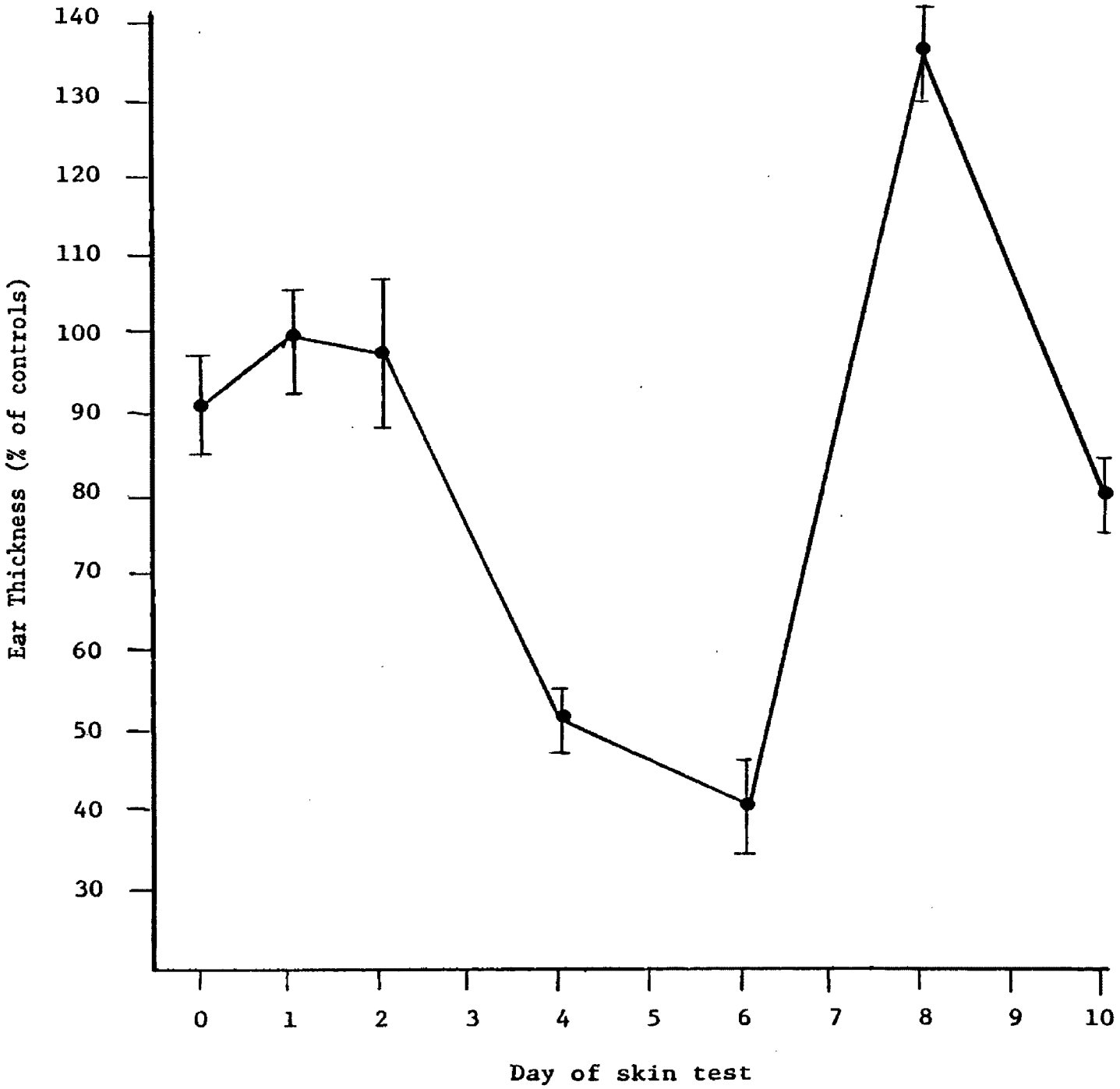


Figure 10. Effect of azathioprine (150 mg/kg administered on day zero) on the DH response of mice previously sensitized with oxazolone. Each point represents pooled measurements from both ears of sensitized mice.

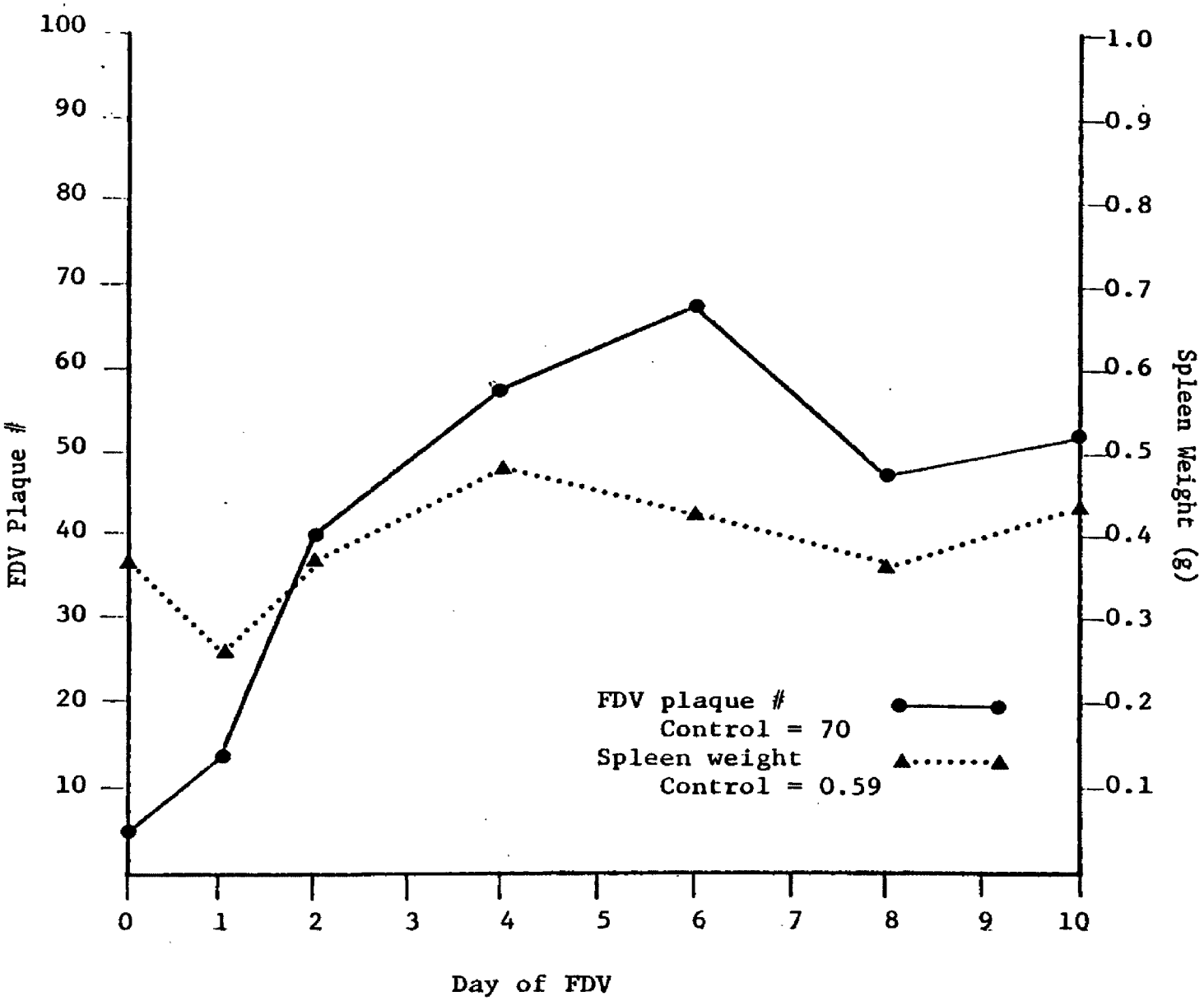


Figure 11. Effect of azathioprine (150 mg/kg administered on day zero) on susceptibility of mice to FDV infection. Each point represents the mean of results from ten mice.

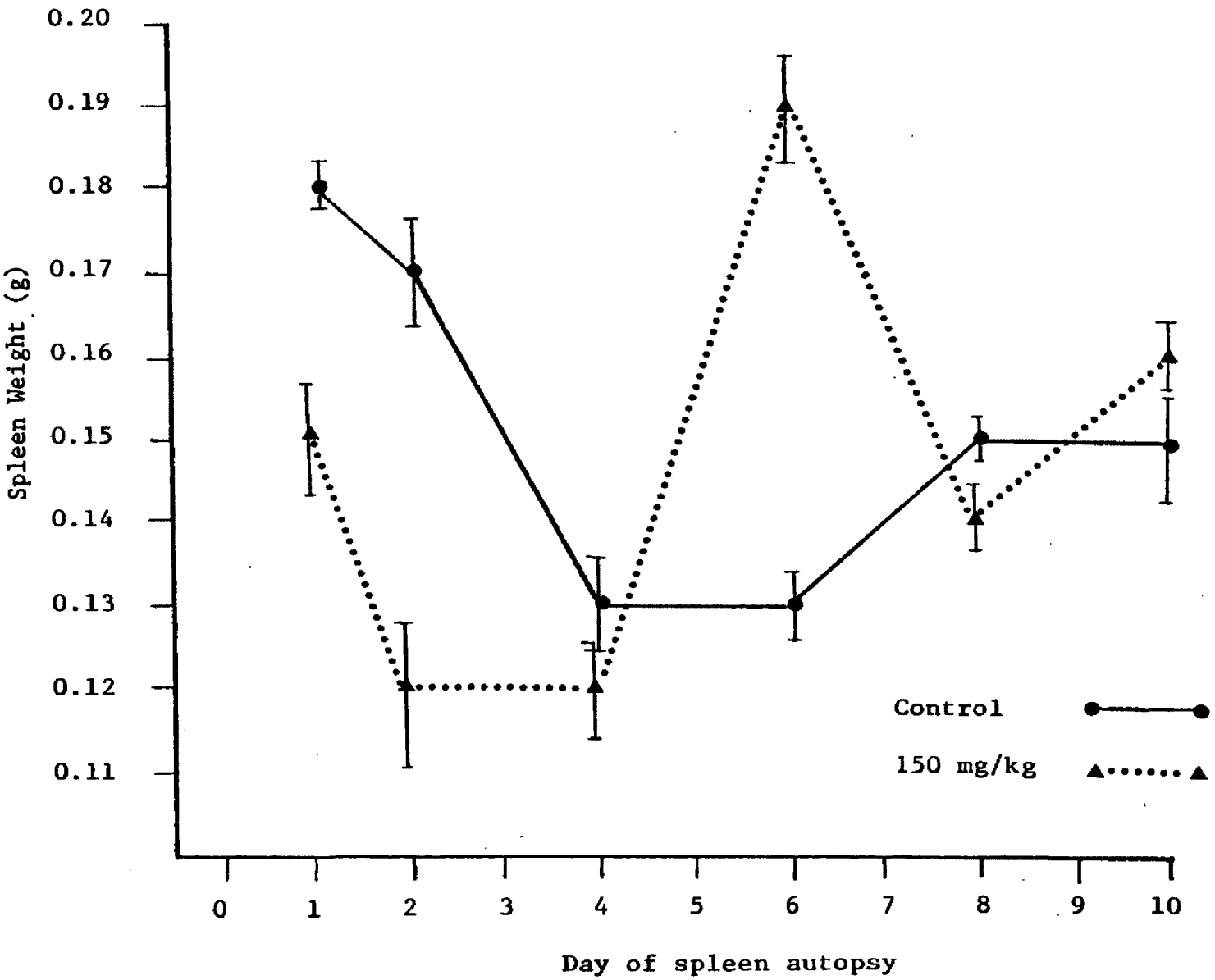


Figure 12. Effect of azathioprine (150 mg/kg administered on day zero) on spleen weights of mice.

Niridazole

The immunosuppressive potential of niridazole, which is relatively nontoxic, was investigated because of reports citing marked suppression of DH to soluble schistosome egg antigens (54). Antibody titer to rabbit red blood cells was decreased by only one 2-fold dilution throughout the interval of drug treatment, which can hardly be considered a suppression of the humoral immune response (Figure 13). Recently humoral immune suppression by niridazole was accomplished by eight days of continued drug therapy (67). One dose of drug produced no noticeable suppression.

The DH response of mice previously sensitized with oxazolone was depressed only slightly between the fourth and sixth days following administration of the drug (Figure 14). However, on the eighth day remarkable enhancement of the delayed response occurred with both high and low doses of drug.

Susceptibility of mice to FDV was not altered by niridazole treatment (Figure 15), a result supported by the fact that humoral immunity was not depressed. Similar to azathioprine-treated mice, spleen weights of niridazole-treated mice peaked during the time of depressed DH (Figure 16).

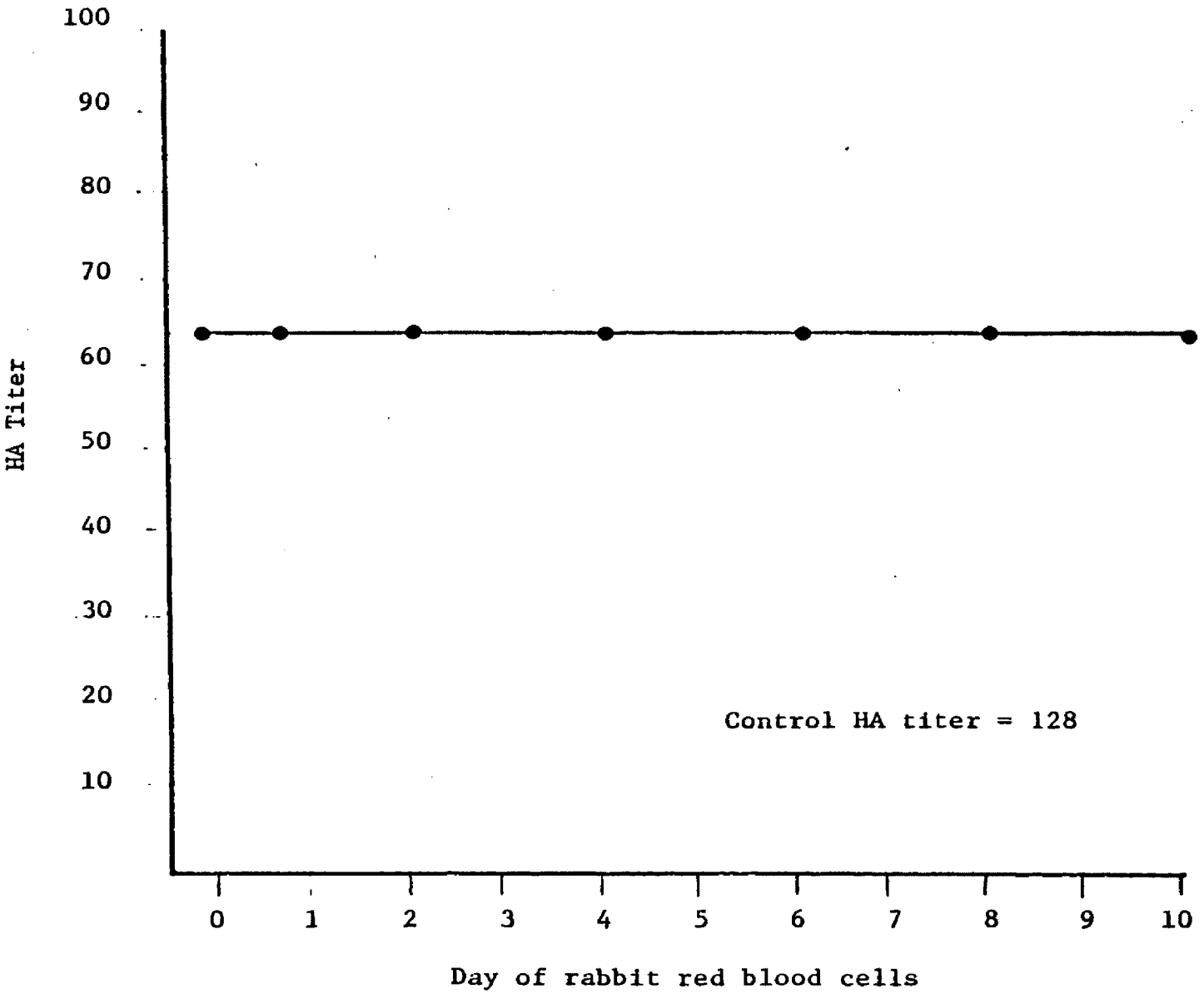


Figure 13. Effect of niridazole (10 mg/kg administered on day zero) on the humoral immune response of mice to rabbit red blood cells. Each point represents pooled sera of five mice. HA = hemagglutination titer.

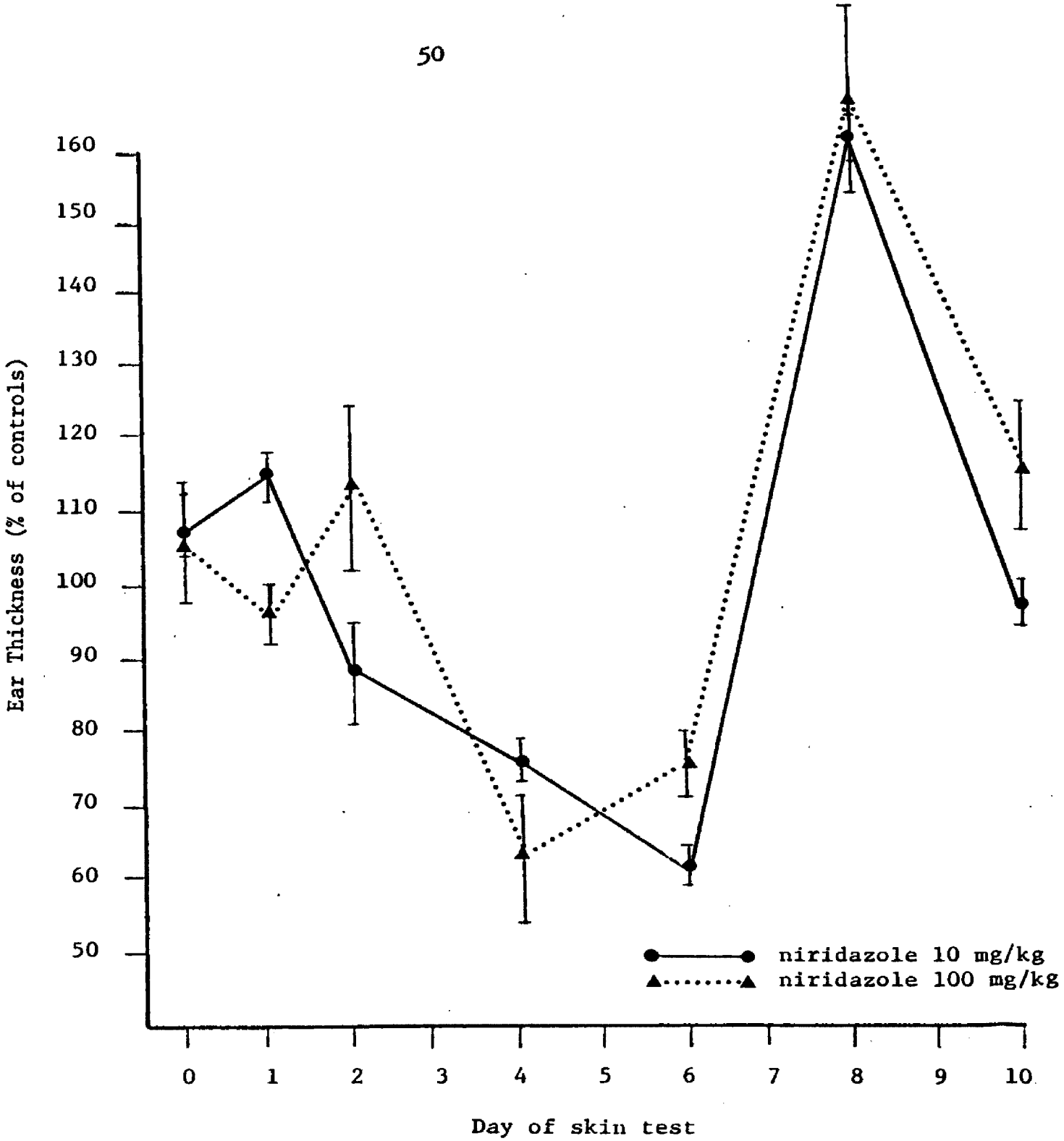


Figure 14. Effect of niridazole (administered on day zero) on the DH response of mice previously sensitized with oxazolone. Each point represents pooled measurements from both ears of sensitized mice.

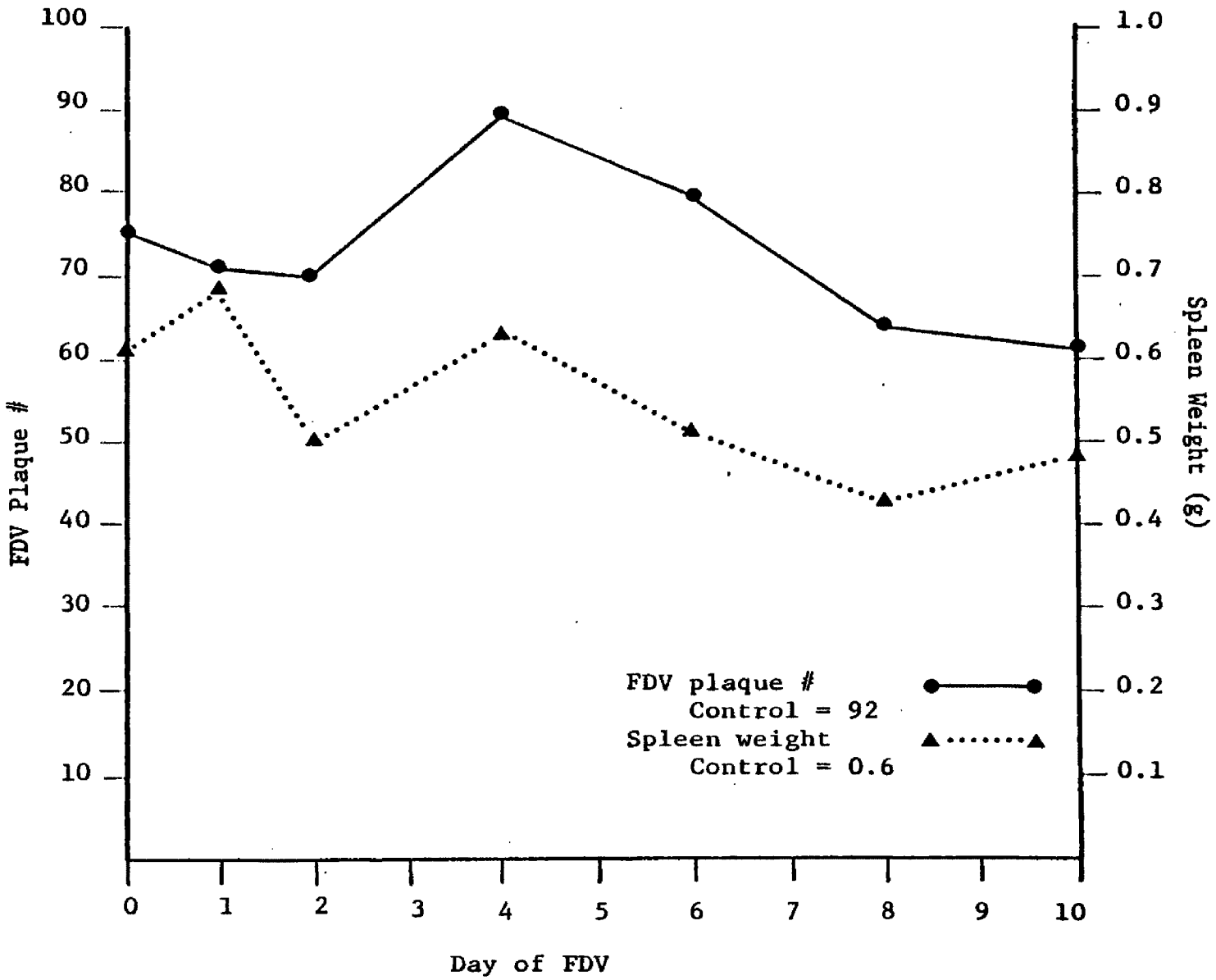


Figure 15. Effect of niridazole (10 mg/kg administered on day zero) on susceptibility of mice to FDV infection. Each point represents the mean of results from ten mice.

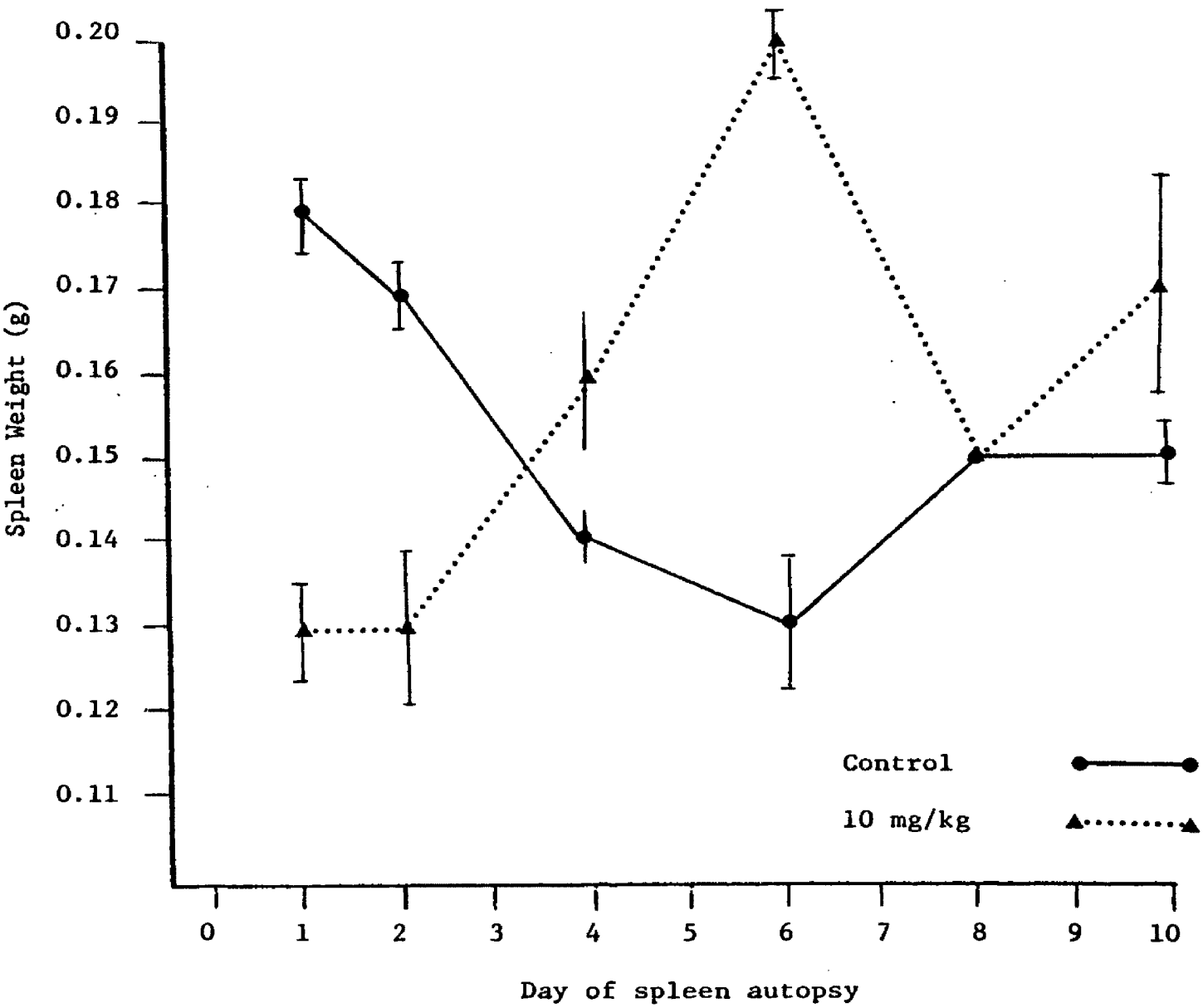


Figure 16. Effect of niridazole (10 mg/kg administered on day zero) on spleen weights of mice.

Methotrexate

The folic acid antagonist methotrexate exhibits considerable toxicity and causes suppression of the humoral immune component of the immune system. Hemagglutination titers to rabbit red blood cells were depressed during the entire ten day interval following drug treatment (Figure 17). In close agreement, susceptibility to FDV was nearly abolished when methotrexate and FDV were given on the same day (Figure 18). Thereafter, susceptibility to infection increased to levels comparable to controls suggesting that B lymphocyte recovery occurred well before recovery of antibody production. A decrease in the population of B lymphocytes in the spleen as assayed by immunofluorescence (Figure 19) correlated with the depressed antibody titer. Spleen weights of drug-treated mice did not vary significantly from controls (Figure 20). Satisfactory data on the effect of methotrexate on the delayed response to oxazolone was not obtained.

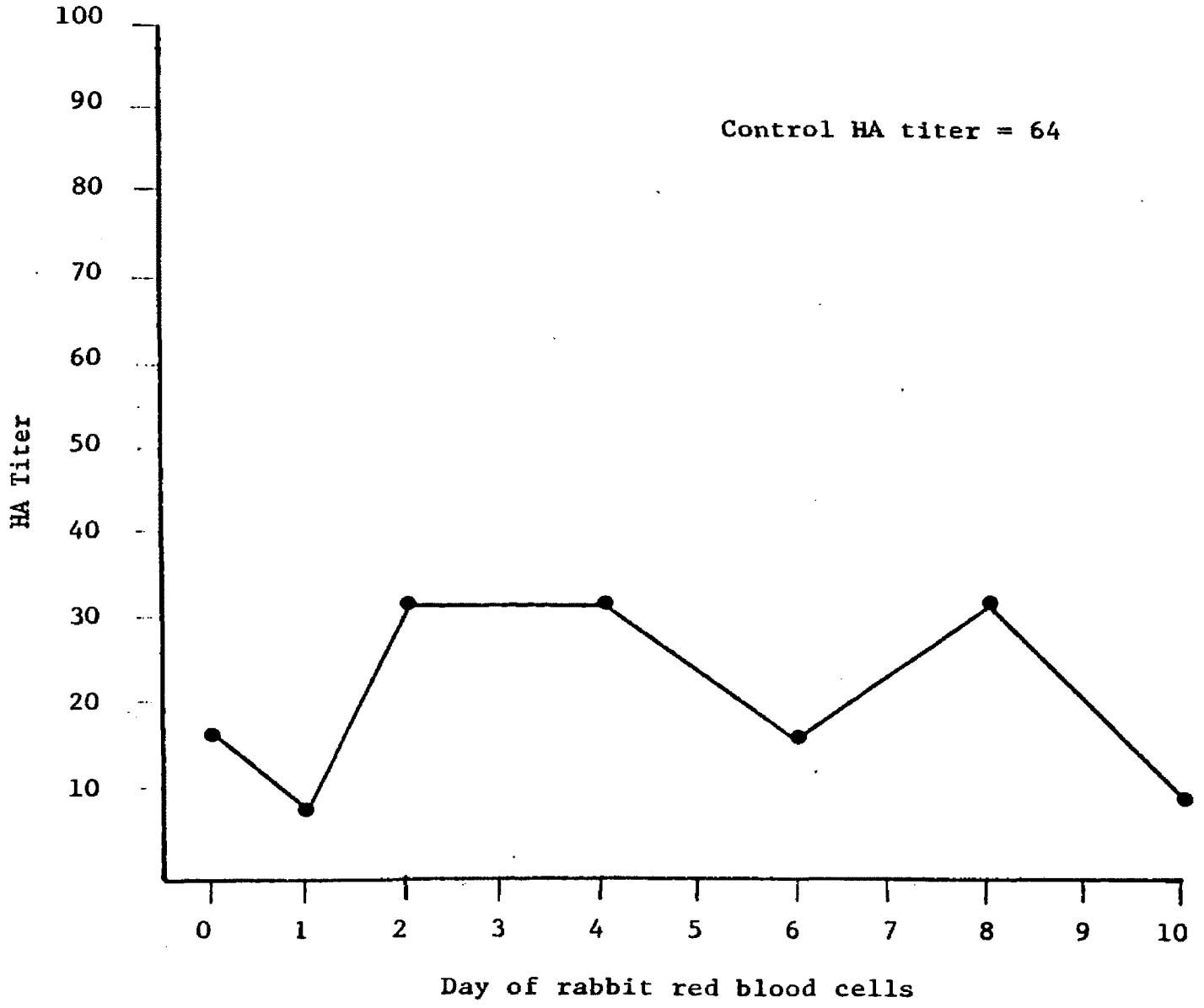


Figure 17. Effect of methotrexate (250 mg/kg administered on day zero) on the humoral immune response of mice to rabbit red blood cells. Each point represents pooled sera of five mice. HA = hemagglutination titer.

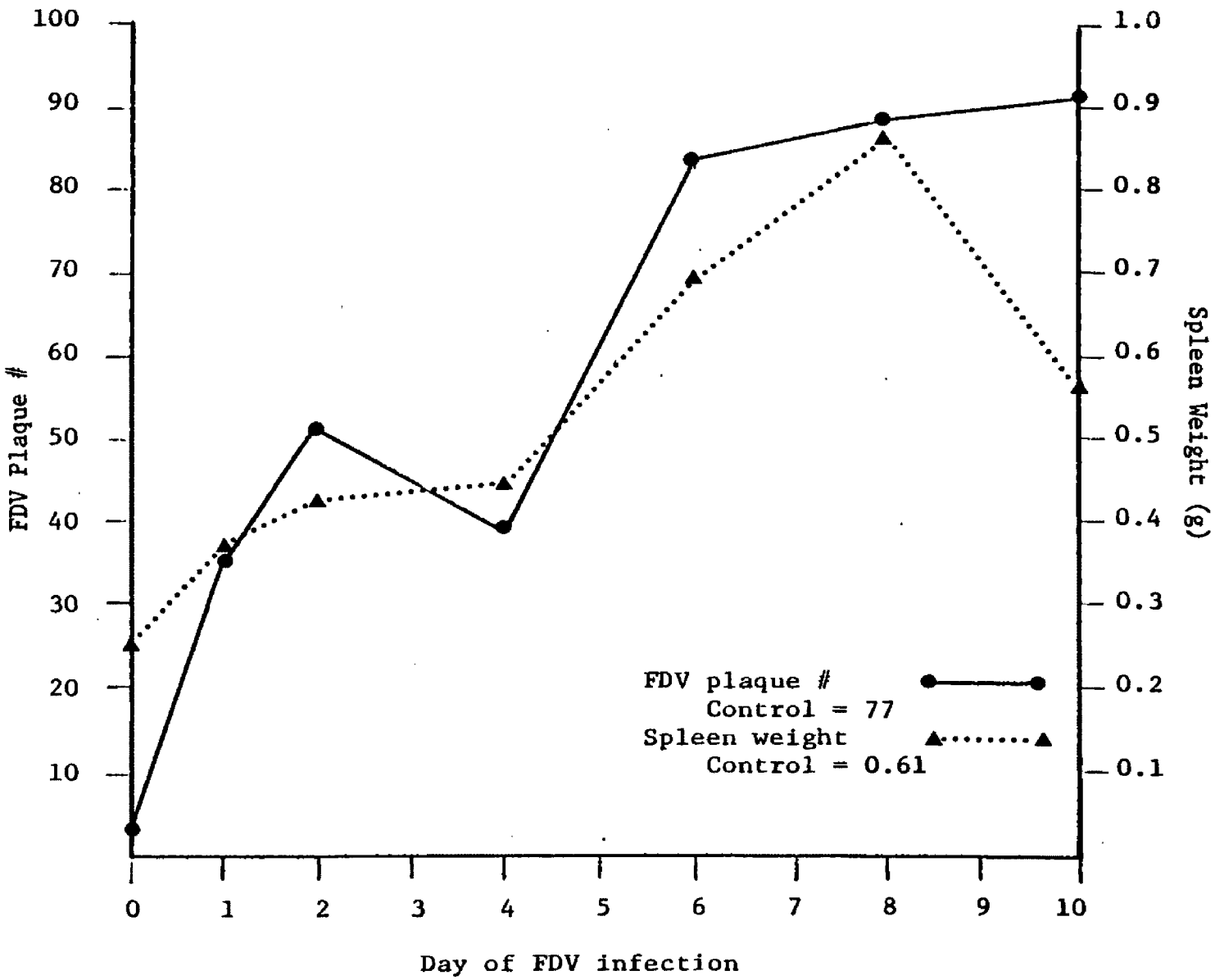


Figure 18. Effect of methotrexate (250 mg/kg administered on day zero) on susceptibility of mice to FDV infection. Each point represents the mean of results from ten mice.

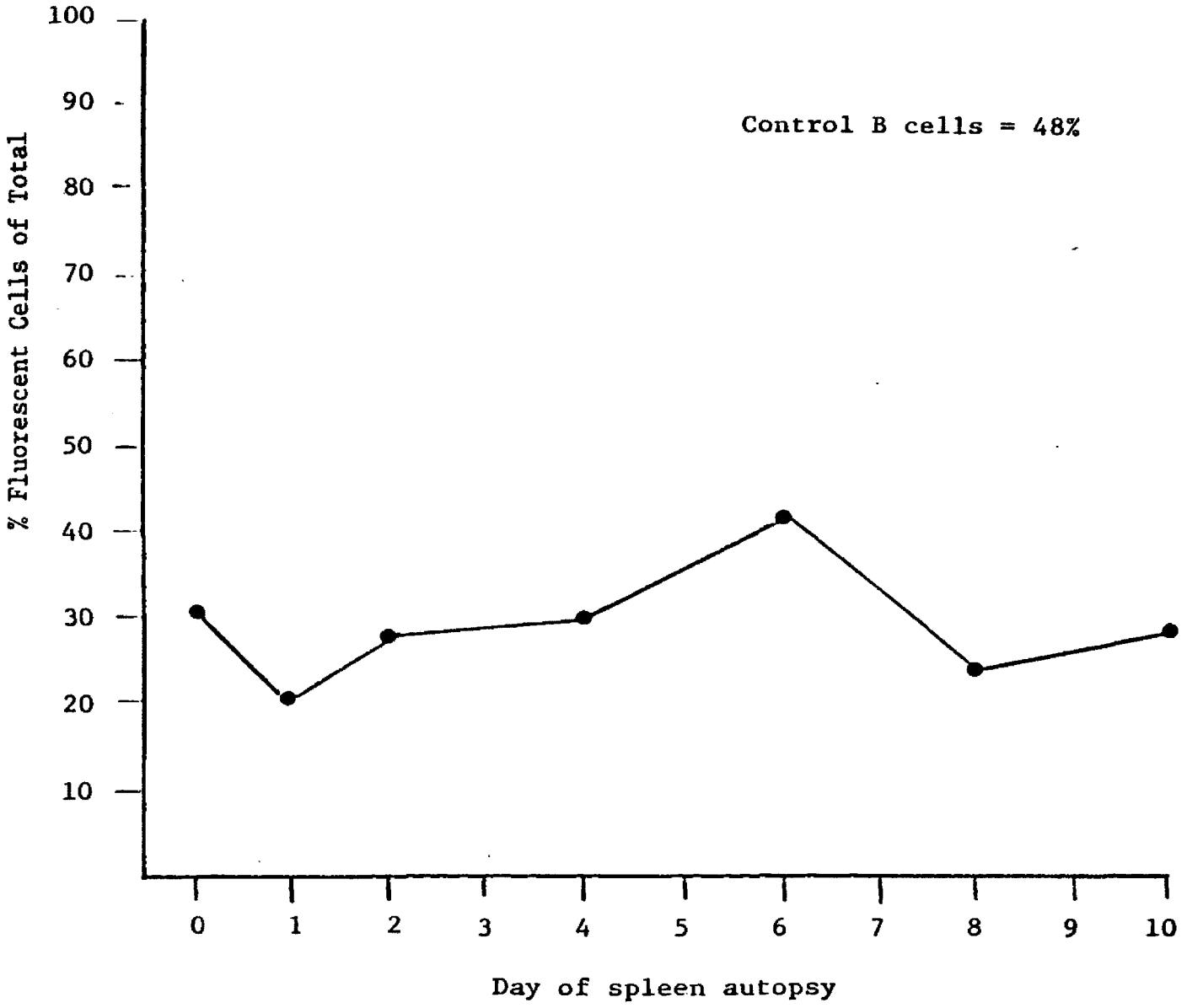


Figure 19. Effect of methotrexate (250 mg/kg administered on day zero) on B cell populations in spleens of mice as assayed by fluorescent antibody.

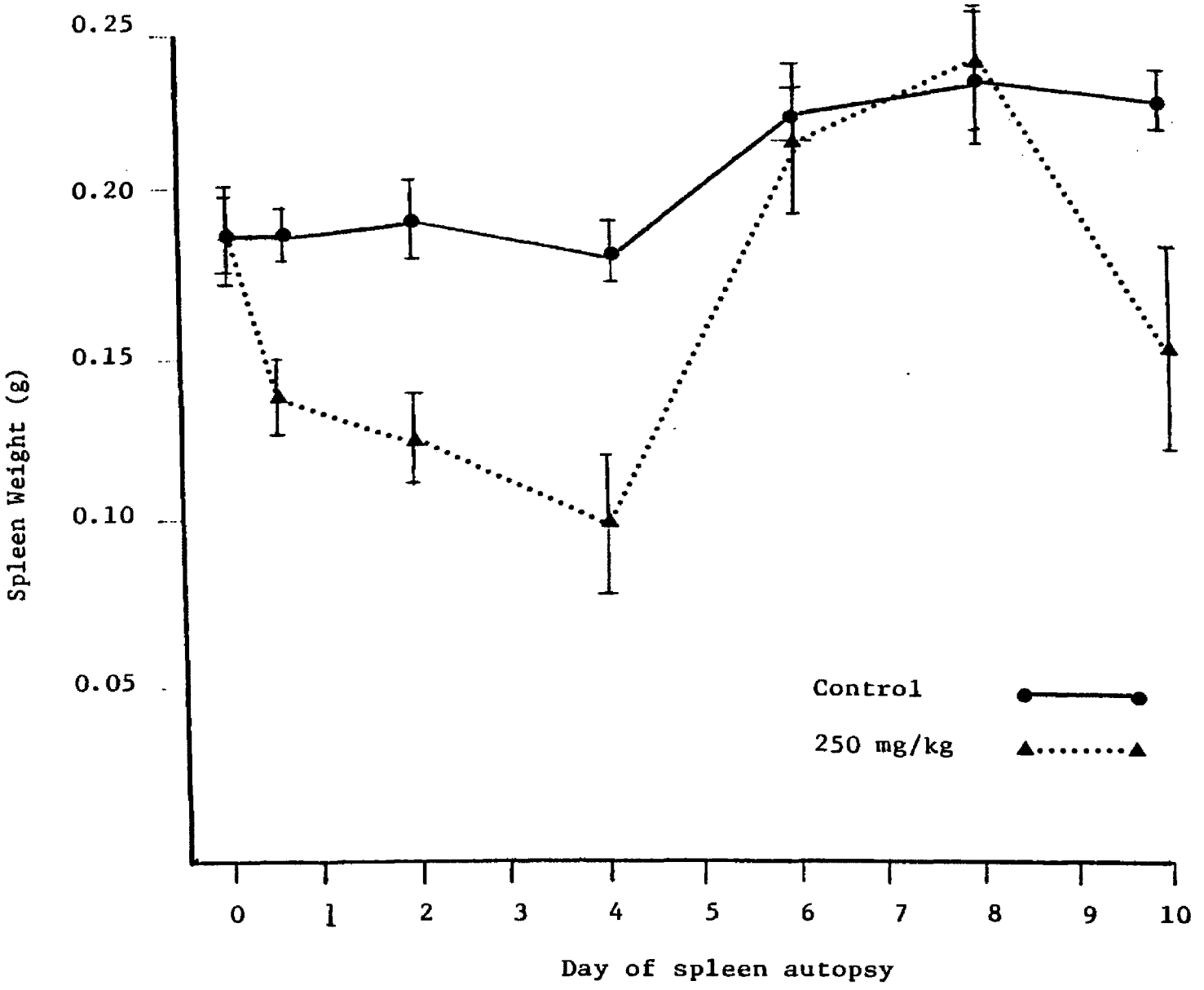


Figure 20. Effect of methotrexate (250 mg/kg administered on day zero) on spleen weights of mice.

Chapter 4

DISCUSSION

Studies of immunodeficiency diseases, the effects of surgical removal of lymphoid tissues, and immunosuppression by drugs have aided in separating the two lymphoid cell populations involved in the immune response. The B lymphocytes are the antibody-producing cells of the humoral immune system, and the T lymphocytes function as helper cells for antibody production to certain antigens and as mediators in cellular immune responses. The T and B cells of animals treated with immunosuppressive drugs during induction of the immune response differ in their sensitivity to various drugs. Therefore, such drugs may affect natural resistance as is evidenced by decreased resistance to infections and tumors in immunosuppressed hosts.

In this study the temporal effects of four drugs on the humoral and/or DH response are investigated. The major emphasis is placed on determining the effects of hydrocortisone acetate. Minimal studies with azathioprine, niridazole, and methotrexate are included as a general reference to determine whether all of these immunosuppressive drugs alter the immune system in a similar manner.

Expression of DH

The effect of cortisone on the afferent and efferent arms

of the delayed response to oxazolone differs with respect to the response at various intervals after administration of the drug. Following the administration of cortisone, expression (efferent arm) of the already established delayed reaction to oxazolone is almost wholly depressed for a period of four days. After an additional two days, the capability of mice to respond to a sensitizing dose partially returns, but complete recovery is not noted until the tenth day. These results suggest several possible effects of cortisone on committed T cells and/or perhaps only on innocent-bystander lymphocytes which are necessary for committed T cells to effect a lesion at the skin test site. One possibility might be that the T cells are directly affected by cortisone and are unable to respond to an eliciting dose of antigen. Another is that some mechanism acts to prohibit migration of the committed cells from the immune cell "reservoir". Lastly, the T-cells may be distributed to other body compartments and become unavailable for expression of DH.

The effect of azathioprine and niridazole upon the efferent arm of the DH reaction does not follow the course observed with cortisone. There is a slight increase in the response on the day following administration of either of these drugs, and on day four and six the response is suppressed. In contrast to the gradual recovery noted in cortisone-treated mice, the response returns to normal or above normal levels in two days.

The mechanism of depression of T cell activity produced by treatment with azathioprine or niridazole may be qualitatively

different than that produced by administration of cortisone. It is essential, however, to point out that cortisone is administered in a carrier which serves to cause a slow release of cortisone from a depot resulting in a prolonged clearance of the drug from the animal. If mice are injected with cortisone alone, the length of the depressed state may be as short as that caused by azathioprine and niridazole. Furthermore, in the form administered, azathioprine and niridazole may be inactive chemically. The means whereby these drugs exert their effect may differ since cortisone may exert its effect directly on lymphocytes while azathioprine and niridazole require chemical alteration in order to exert their depressive properties. The recovery of the DH response would, therefore, occur more abruptly since altered chemicals may be rapidly eliminated from the animal. Additional studies on the status of circulating lymphocytes following administration of azathioprine and niridazole hopefully will provide insight into the similarities or differences between these drugs and cortisone.

Induction of DH

In the induction (afferent arm) studies, separate groups of mice are treated with cortisone at various intervals during a ten-day period and sensitized with oxazolone on the final day of drug administration. After an additional seven days the contact sensitivity assay is performed. In animals given cortisone two days before or at the time of exposure to oxazolone, the DH response is depressed. However, unlike the effects of cortisone on the expression of the DH response, mice treated with cortisone, sensitized

with oxazolone four or six days later, and skin tested after an additional seven days, respond with increased DH reactivity compared to untreated control animals. As mentioned previously, this prolonged state of depression may be attributed to the "depot" effect. It is of interest to observe that in the tests performed to determine the influence of cortisone upon either the afferent or efferent arm of the DH reaction to oxazolone, the animals recover their ability to respond normally about ten days after receiving cortisone. Taken at face value this observation indicates that T cells recover their ability to become sensitized and to produce lymphokines at the same time. There must, however, be a fundamental difference in the recovery of competent and committed T cells since it requires a period of about four days before competent cells are sufficiently activated to produce a state of delayed hypersensitivity (2).

Induction of Humoral Immunity

The effect of hydrocortisone acetate on the afferent arm of the humoral immune response indicates that T and B cells are differentially depressed by the drug. Although the antibody response of mice to rabbit red blood cells (a T-cell-dependent antigen) is depressed during the entire interval of study, the response to LPS (a T-cell-independent antigen) is depressed only on the first or second day following administration of cortisone. During these two days the spleen size is markedly reduced, and in most animals, lymphocytes can not be obtained even from pooled spleen samples. On the following days, however, the spleens begin to "regenerate", and B cells, assayed by immunofluorescence, are detected in sus-

pensions of spleen cells. The depression of the antibody response to LPS is undoubtedly due to temporary functional absence of spleen lymphocytes and circulating B cells. B cells may be driven into another body compartment, such as the bone marrow (35), but obviously are not present in the spleen. On the other hand, B cells are available even though nonfunctional since it is observed that drug-treated mice are as susceptible to infection with FDV as are untreated controls. However, the T lymphocytes appear to be affected in a different manner. Depression of the antibody response to red blood cells suggests that T cells may be sequestered elsewhere in the body and are not available for contact with antigen or that cortisone alters antigen-recognition thus preventing stimulation of B cells for antibody production.

The mechanism of action of azathioprine and methotrexate differs from that of cortisone and from each other. The antibody response to rabbit red blood cells is depressed following treatment with either drug. However, azathioprine depresses the antibody response only when the drug is administered on the same day or one or two days prior to injection of antigen, whereas methotrexate depresses the humoral response throughout the entire period of study. On the other hand, susceptibility to infection with FDV decreases during the first two days following administration of either drug but is more persistent with methotrexate. These results indicate that B lymphocytes are either unable to respond to antigen or are unavailable for contact with antigen. The similarity in response of mice given azathioprine and methotrexate to infection with FDV and the difference in humoral antibody suppression in-

dicates that azathioprine is catabolized more rapidly. The B cells are susceptible to FDV by eighteen days following drug treatment but respond to antigenic stimulation four days after azathioprine treatment and some time past ten days following methotrexate.

Niridazole, however, apparently acts by an entirely different mechanism than that of the other drugs since no depression of antibody response or FDV susceptibility is observed.

Since macrophage function was not investigated in this study, further experiments might indicate whether the depression of DH and humoral immunity could also be attributed to the inability of macrophages to process antigen or respond to MIF.

Cortisone

The function of immunosuppressive drugs upon the two types of lymphocytes is variable, and the temporal effects on either B cells, T cells, or both will be reflected in the immunological response of the host. As shown by other investigators (23, 26, 40), the action of glucocorticosteroids on the cellular and humoral immune responses is dependent upon the effect on the two populations of lymphoid cells. Corticosteroids are also used to suppress non-specific inflammatory reactions, and are of value in controlling allograft rejection. The DH response is a combination of afferent and efferent processes. Macrophages process antigen and, in turn, stimulate T lymphocytes. These sensitized lymphocytes subsequently release chemotactic factors that, in addition to other functions, inhibit macrophage migration.

Corticosteroids suppress DH although the mechanism of action

is unclear. According to Claman (23) and de Sousa and Fachet (29) lymphocytes in the cortex of the thymus are steroid-sensitive. However, these cells become resistant during migration through the thymus medulla and maintain this resistance during uncommitted and committed stages of differentiation. In the mouse system a high incidence of lysis of the thymus cortical lymphocytes is observed as early as four hours following cortisone acetate administration (14, 29). By 24 hours cytolysis is complete and all nuclear debris is removed from the cortex. In contrast, human and guinea pig thymus cells are relatively resistant to *in vitro* lysis by hydrocortisone (24), indicating the species variability to drug suppression. Of interest is the observation that the cells subject to lymphocytolysis are not immunocompetent since thymus cells from cortisone treated mice are more active in initiating GVH reactions than are thymus cells from untreated animals (25).

Using PHA-induced lymphocyte transformation as a measure of cell-mediated immunity, Webel et al (103) report maximal suppression of blastogenesis of peripheral lymphocytes within four hours following a single dose of methylprednisolone to human volunteers. Gradual return to normal lymphocyte transformation is apparent by 72 hours. On the other hand, pokeweed mitogen stimulation of lymphocytes from steroid-treated subjects is not significantly impaired, suggesting a differential suppression of T and B cells.

As shown by Casey and McCall (20) using the macrophage migration test, the afferent arm of the DH response is inhibited when rabbits are treated with steroid at the time of sensitization with

BCG. If steroid treatment is delayed until BCG sensitization has developed, inhibition of macrophage migration occurs. Furthermore, when BCG-sensitized lymphocytes are added to cultures of cells obtained from steroid-treated rabbits the inhibition of macrophage migration is reestablished. Other researchers (7, 104) conclude that steroids do not block antigen processing by macrophages or synthesis of MIF by sensitized lymphocytes. Balow and Rosenthal (7) suggest that corticoids interfere with the macrophage response to MIF, perhaps via some alteration of the macrophage itself. Such an alteration might be stabilization of the macrophage cell membrane which could prevent secretion of the macrophage lysosomal enzymes (82).

Further data (73, 95) also indicates that macrophage-monocyte defects may contribute to the steroid-mediated suppression of cellular immunity which is apparent in patients receiving corticosteroids and manifested as increased susceptibility to infectious agents. Monocytes are those circulating cells which respond to chemotactic stimuli, such as lymphokines or products of infectious agents, and mature into macrophages at sites of infection or inflammation. Corticosteroids may in fact interfere with interaction of monocytes and chemotactic stimuli thus inhibiting some trigger of crucial metabolic events.

In a recent series of papers (91, 92, 93) Stevenson refers to a polymorph migration stimulator factor (PMS) which is released from steroid-treated macrophages. Apparently monocytes interact with steroid <u>in vivo</u> and liberate PMS <u>in vitro</u> resulting in polymorph migration. The migration is dependent upon the presence

of monocytes but not lymphocytes in the test chamber. Serum from steroid-treated patients does not stimulate polymorph migration until the euglobulin fraction is removed. A release of polymorphs from the bone marrow may be induced by PMS resulting in the increase of these cells in the circulation following corticosteroid treatment. Since steroids prevent aggregation of polymorphs at inflammatory lesions (29), Stevenson suggests that PMS may induce functional changes in polymorphs which prevent their response to lymphokines or chemotaxins. In addition, he also indicates that PMS may not serve a functional role in preventing migration of polymorphs but that another mechanism might be responsible for preventing the response of these cells to the soluble mediators of inflammation.

Parrott et al (65) stress the importance of T cells in induction and expression of contact sensitivity in mice. According to de Sousa and Fachet (29), cortisone administered prior to a sensitizing dose of oxazolone to the ear skin does not eliminate T cells in the draining lymph node but does prohibit transformation of these lymphocytes. When cortisone is administered after sensitization, transformation of T cells occurs normally, and the large pyroninophilic blast cells are not affected. Therefore, these investigators suggest that the value of cortisone as an immunosuppressive agent of contact sensitivity depends upon time and the dose of drug that will impair T cell transformation in the draining lymph node. They concede, however, that the impairment may be due to inhibition of polymorph and macrophage infiltration at the site of oxazolone application. Cortisone may also prevent antigen release

from epidermal cells or antigen transport to the draining lymph node.

Less work has been done on the effects of steroids on humoral immunity. In general, antibody production is inhibited if cortisone is administered prior to antigenic stimulation. For example; Claman (23) proposes that B lymphocytes are steroid-sensitive prior to contact with antigen but become steroid-resistant following differentiation into committed B cells. Elliott and Sinclair (32) show that both 19S and 7S antibody are maximally suppressed when cortisone is given three to four days prior to sheep red blood cells. However, only 7S antibody is suppressed when cortisone is given after the antigen. A striking decrease in the peripheral lymphocyte pool also occurs during suppression of the antibody response. Although the necessity of circulating lymphocytes for expression of humoral immunity is well established (44), defects of antigen trapping and processing by macrophages, and antigen retention in lymphoid organs should not be overlooked as possible causes of decreased antibody levels. As shown by Petranyi et al (69) induction of memory and synthesis of 7S antibody appears more susceptible to a single large dose of cortisone. However, when cortisone is given from five days prior to antigen to the day of antigen, enhanced 19S antibody is apparent during 7S suppression. As a possible explanation they suggest that cortisone acts preferentially on the antigen-sensitive precursor cells. Since there are 15 times less 7S precursor cells than 19S cells in the bone marrow, this may explain the pronounced 7S antibody suppression. Also 7S feedback-inhibition of 19S antibody production may allow for the enhanced IgM production.

According to Cohen and Claman (27), the B cell precursors are more susceptible to suppression than are the helper T cells. Spleens of hydrocortisone-treated mice are deficient in antibody-forming cells, and immunocompetence may be restored by repopulating the circulatory system of these mice with bone marrow but not with thymus cells. These researchers suggest that antibody-forming precursor cells in the bone marrow and the committed B cells are corticosteroid-resistant, while immunosuppression of the humoral response occurs at the level of the antigen-reactive B cell.

In contrast to other data, Butler (17) reports a steroid-induced decrease in ongoing IgG synthesis in humans. He hypothesizes that the drug inhibits the committed plasma cell and does not affect induction of primary or secondary antibody responses.

Another possible explanation of corticoid immunosuppression involves redistribution of circulating lymphocytes into other body compartments or arrest of lymphocyte recirculation rather than corticoid-induced lymphocyte alteration. Fauci (35) demonstrates the accumulation in the bone marrow of functionally mature lymphocytes bearing T and B cell markers following steroid treatment in guinea pigs. A similar effect is seen by Cohen (26) using mice and by Yu et al (108) using human volunteers.

Azathioprine

Azathioprine is used extensively in the treatment of patients undergoing organ transplantation or having autoimmune disease. This drug is an imidazole derivative of 6-MP and is not immunosuppressive

until it undergoes cleavage by sulfhydral compounds to release 6-MP. Depletion of sulfhydral or amine groups during the formation of 6-MP may contribute to the immunosuppressive effects of azathioprine. The drug is broken down to its active form by erythrocytes and white blood cells and has a serum half-life of about four hours (31).

Immunosuppressive effects of azathioprine on the cellular immune responses vary considerably with species, antigen and dosage although cellular immunity is not generally depressed. For example, human volunteers receiving low doses of azathioprine elicit normal induction and expression of DH to simple chemicals such as poison ivy allergen, dinitrochlorobenzene (DNCB) (52), and keyhole limpet hemocyanin (94). The DH response to DNCB in drug-treated guinea pigs is not suppressed (53). On the other hand, the DH response to tuberculin, diphtheria toxoid and serum albumin is reduced or delayed in a variety of animals treated with this agent (77). Multiple dose azathioprine therapy prolongs allogeneic skin graft survival in rats (79), suggesting suppression of the cellular immune response. In general, suppression of the graft rejection mechanism is more effective when the drug is administered following antigenic stimulation. From all of these data it appears that DH to simple chemicals is more difficult to suppress.

Poulter et al (70) report reduction in thymic weight and in numbers of lymphoid cells bearing theta antigen in mice treated with azathioprine. The population of theta-positive cells is depleted for three to six days following drug treatment. As in the studies using cortisone, T lymphocytes in the thymus cortex are azathioprine-

sensitive while the T cells of the thymus medulla are resistant. These authors suggest that azathioprine either deplete certain populations of lymphocytes or alters the antigenicity of the antigen-reactive T cells. Bach and Dardenne (5) note a selective suppression by azathioprine of the thymus-derived rosette forming cells. The ability of lymphocytes to form rosettes is an *in vitro* assay for detection of cells bearing theta antigen (6). The incorporation of tritiated thymidine into mixed lymphocyte cultures is decreased when the drug is introduced into the cultures during the first 24 hours (4), indicating that this agent may alter antigen recognition during the early phase of immune induction. Using labeled lymphocytes to observe lymphoid cell migration in mice, other investigators find no evidence of cytolytic activity by azathioprine (38). Although the drug is most effective against cells actively synthesizing DNA (103), Fournier et al (38) show that cell proliferation is not suppressed, since lymphocyte migration does not involve proliferation. Suppression of migration of lymphoid cells to the lymph nodes suggests that azathioprine selectively affects the short-lived recirculating T lymphocytes (58). The inductive phase of the immune response is apparently more susceptible to suppression since drug-treated mice acquire resistance to *Listeria monocytogenes* following the passive transfer of immune lymphoid cells (97). Although the mechanism of azathioprine-induced immunosuppression is still unclear, a membrane change involving the antigen receptors probably offers the most reasonable explanation.

In contrast, other investigators (48) indicate no reduction

of rosette forming T cells, casting some doubt on the assumption of azathioprine-induced alteration of lymphocyte antigenicity.

Azathioprine may also suppress the primary antibody response. By using a multiple dose regimen, Santos and Owens (78) report a depression of the antibody response to sheep red blood cells when drug treatment is begun one, two, or three days prior to antigen administration or four days following the antigen. However, maximum suppression occurs when therapy starts on the second day prior to an injection of sheep red blood cells. In addition, the suppression is specific since restimulation with the same antigen elicits no antibody response, but a primary response to a different antigen is mounted. This may indicate an inability of committed T cells to function as helper cells in antibody production. Selective suppression of 7S antibody occurs with prolonged or enhanced synthesis of 19S antibody, suggesting that azathioprine may function at the level of the mechanism resulting in the switch from an IgM to an IgG-producing B cell (52, 94). Occasionally enhanced antibody production is noted in azathioprine-treated subjects (22, 94). If azathioprine is cytotoxic to lymphoid cells, nucleic acid may be released when the cells lyse. Nucleic acids are absorbed by living cells and stimulate cell division and protein synthesis (8). Therefore, immature lymphocytes may incorporate the nucleic acid and be transformed into antigen-reactive cells.

Azathioprine has little effect on the secondary antibody response indicating that memory cells are resistant to suppression by this drug (106).

Several workers also suggest that azathioprine may exert

an anti-inflammatory action. Borel and Schwartz (15) note suppression of the passive Arthus reaction, and Page and Condie (64) indicate that neutrophils appear at the inflammatory site but normal migration of lymphocytes does not follow. Perhaps chemotactic substances are not produced by the neutrophils, or mononuclear cells may be either destroyed or unresponsive.

Niridazole

Niridazole is a drug used in the treatment of helminthic infections such as schistosomiasis. Mahmoud et al (55) report that a single low dose of niridazole suppresses granuloma formation around Schistosoma mansoni eggs in infected mice. Repeated daily doses prolong allograft survival in mice when drug treatment is begun either before or after grafting. Interpretation of these results suggests that niridazole therapy produces prolonged suppression of cellular immunity. Consequently, when the granulomatous response is decreased, eggs mature and hatch, thus facilitating the extraction of adult worms. Niridazole is converted in vitro to water soluble metabolites which bind to serum proteins, and this conversion may account for the extended suppression (33). More recently, Daniels et al (28) report that a niridazole metabolite found in serum suppresses the DH response of guinea pigs sensitized with o-chlorobenzoyl-bovine gamma globulin. This metabolite prevents MIF production by lymphocytes but does not affect the response of macrophages to preformed MIF. Lymphocyte incorporation of tritiated thymidine is unaffected in niridazole-treated guinea pigs. An earlier study by Mousa et al (62) is

contradictive, in that they find repeated injections of a high dose of niridazole do not alter established schistosome infections. However, the therapeutic value of niridazole is increased when the drug is given prior to infection.

Decreased bone marrow and white blood cell counts and increased susceptibility to infection are not noted following treatment with niridazole (41, 86).

The influence of this drug on humoral immunity has not been extensively studied, but Pelley et al (67) indicate that repeated doses of niridazole suppress antibody production for a short duration. Although the mechanism of action of niridazole is not understood, several investigators (72) suggest that suppression of nonspecific inflammatory reactions is involved.

Methotrexate

Methotrexate (amethopterin) is an antimetabolite which functions by competing with folic acid for the enzyme dihydrofolate reductase. Thus, DNA synthesis is impaired by blocking the major pathway leading to thymidine formation (66). The drug is excreted or metabolized within four hours after administration, but a threshold amount of the metabolites may persist for up to three weeks in tissues containing high concentrations of folic acid (37).

In some cases methotrexate prevents development of DH. For example, contact sensitivity to DNCB in man (59) and to PPD in guinea pigs (107) are suppressed. However, this drug apparently does not reverse positive skin tests for established DH (47). Methotrexate does not prevent the development of immunoblasts in

lymph nodes following exposure to contact sensitivity agents as incorporation of tritiated thymidine occurs normally (30). Inhibition of induction of DH seems to occur at the level of the small lymphocytes which develop from immunoblasts. However, Kessel et al (50) report that normal and leukemic small lymphocytes are methotrexate-resistant, but granulocytes take up the drug. Furthermore, a single dose of the agent has no effect on skin graft survival in rats, but multiple doses suppress graft rejection when drug therapy is initiated five to seven days after grafting (79). Methotrexate has no effect on the established resistance to the intracellular parasite Listeria monocytogenes, indicating that the suppression is not directed at macrophages (98).

Methotrexate is more effective in suppressing humoral than cellular immunity. Administration of multiple doses of the drug starting one or two days prior to injection with sheep red blood cells significantly prolongs primary antibody induction and suppresses the peak titer (78). The 7S antibody response is selectively suppressed when methotrexate is given from two days before to two days after the antigen (80). With peripheral blood mixed lymphocyte cultures, tritiated thymidine incorporation is inhibited up to four days after beginning the culture (4), suggesting a possible effect on proliferation of both T and B cells. When the drug is given four to seven days prior to injection of the antigen, immunity is actually enhanced as noted by marked lymphoid hyperplasia (98). Although the mechanism of enhancement is unclear, this phenomenon implies that inadequate immunosuppressive therapy may in fact enhance immune responses. Maley and Maley (57) suggest that following methotrexate

treatment, an increase in thymidylate synthetase, an enzyme necessary for the conversion of deoxyuridylate to deoxythymidylate, may account for the enhanced immunity by actually stimulating cell proliferation.

Overview on the Effects by these Four Agents

Data obtained from the cortisone studies in this investigation are in agreement with previous results indicating that both cellular and humoral immunity are suppressed. According to de Sousa and Fachet (29), transformation of committed T cells is prevented when cortisone is given prior to a sensitizing dose of oxazolone, but DH develops normally when cortisone is given after T cell transformation is initiated. In this study transformation of committed T cells is inhibited when cortisone is administered from zero to four days prior to oxazolone sensitization. Drug treatment at earlier times allows development of the DH response in four days as expected (24). However, as evidenced by the experiment concerning the effect of cortisone on the afferent arm of the DH response, uncommitted T cells are not affected by cortisone and undergo normal transformation after a sensitizing dose of oxazolone to give maximum expression of DH in four days. Therefore, this study suggests that cortisone exerts differential suppression on the population of committed T cells. With this in mind, cortisone suppression of macrophage function seems doubtful since macrophages are involved in both induction and expression of DH. Cortisone-induced alteration of antigen recognition offers the most probable explanation of the mechanism of action of this drug. Studies

using passive transfer of sensitized cells would aid in understanding the mechanism.

The effects of cortisone on humoral immunity are in partial agreement with this speculation. Previous investigators note that cortisone suppresses blastogenesis to the T-cell mitogen PHA but not to the B-cell mitogen pokeweed (103). Similarly, suppression of the antibody response to the T-cell-dependent red blood cell antigen is more pronounced than suppression of the response to the T-cell-independent LPS antigen. Again, an alteration in antigen recognition by T cells is suggested, but in this case, the uncommitted T cells are involved.

According to the literature, azathioprine and niridazole also suppress cellular immunity. Suppression of the established DH response by azathioprine from two to six days following the drug administration corresponds with the absence of theta-bearing T cells noted by Poulter et al (70) during this time. Azathioprine is metabolized and excreted rapidly. Thus, the action of this drug on only a small percentage of the committed T cells may explain the nominal suppression and rapid recovery of the DH response observed in this study. A single dose of niridazole also causes short-term suppression of the efferent arm of the DH response which is similar to the suppression noted with azathioprine. In both instances alteration of antigen recognition or cytolysis of committed T cells is implicated. The uncommitted T lymphocytes are apparently unaffected and may respond to antigenic stimulation following elimination of the drug.

The B cell function during development of humoral immunity seems to be resistant to suppression by these two drugs. In fact, the antibody response may even be enhanced by azathioprine. A cytotoxic effect on lymphocytes could account for this enhancement by releasing free nucleic acid as described earlier (8). The actual mechanism of suppression by these drugs is difficult to assess with this study because committed T cells are examined in the expression of DH and uncommitted T cells are examined in the induction of humoral immunity.

Methotrexate apparently exerts an entirely different mechanism of action than the other three drugs and suppresses the B cell compartment of the immune response. On the basis of humoral immunity alone, antibody production and actual numbers of B cells are reduced, suggesting suppression of lymphocyte proliferation.

Immunosuppressive agents may act cooperatively or antagonistically with immunological defenses, and evidence has accumulated that interference with the cell-mediated or humoral immune systems may augment one component while depressing the other. Cyclophosphamide therapy results in depression of the entire immune response initially but continued depression of humoral immunity is accompanied by potentiation of cell-mediated immunity (49). Conversely, sheep red blood cells and LPS antibody responses in Pseudomonas aeruginosa infected mice are enhanced during depression of contact sensitivity of oxazolone (18). Petranyi et al (69) find that variations in dose and time of drug administration alter the antibody response and induce selective enhancement of 19S antibody.

The T and B cells exert feedback inhibition, and circulating antibodies may inhibit development of cell-mediated immune responses by activating suppressor T cells (42). As a result, active T cell proliferation ensues. Immunosuppressive drugs may eliminate feedback inhibition and suppressor cell populations, resulting in enhancement rather than suppression of immune responses.

Expanded knowledge of temporal effects of immunosuppressive drugs has important practical applications in determining drug regimens in transplant cases and tumor therapy. Differential suppression by drugs might modulate the balance between cellular rejection mechanisms and humoral antibody enhancement. Although it is difficult to make generalizations about drug treatment since dose, schedule, route of administration, individual variability, and specificity of lymphoid cell populations introduce considerable variation, it is reasonable to expect that rational drug management may modulate the immune response in a practical fashion for human disease treatment.

Chapter 5

SUMMARY

The ultimate role of immunosuppression has been to control undesirable immune responses without altering the essential immune mechanism. However, to initiate reasonable therapeutic regimens, the effects of immunosuppressive drugs on the normal immune responses should first be determined. In this study the temporal effects of four drugs on primary antibody production and/or delayed hypersensitivity (DH) were examined. With respect to the time of drug administration, results indicated that lymphoid cell differentiation was accompanied by changes in susceptibility to various chemical agents.

Hydrocortisone acetate, a corticosteroid with anti-inflammatory properties, suppressed both cellular and humoral immunity. The effect of hydrocortisone on the established DH response to oxazolone was more prolonged and severe in comparison with the drug's effect on induction of the DH response, but this may be explained on the basis of time involved for drug catabolism. Induction of antibody production in treated animals indicated that T lymphocytes were more susceptible than B lymphocytes. Since unsensitized T cells, memory T cells, and B cells appeared to be affected by hydrocortisone, either inability of lymphocytes to respond to antigen or sequestration of antigen-reactive lymphocytes in some other body compartment was suggested.

Azathioprine and niridazole suppressed the established DH response, but the effect did not occur immediately as with cortisone. This suggested that an interval of drug modification was required to render the drugs "active". In addition, azathioprine also caused a brief suppression of antibody production. Apparently drug modification required four days, since suppression of DH and humoral immunity occurred four days following drug treatment. During this interval antigen-sensitive lymphocytes were able to differentiate and proliferate. Therefore, because recovery of immune responses occurred promptly following elimination of drug from the system, azathioprine and niridazole were probably cytotoxic to the sensitized lymphocytes and did not inhibit cell proliferation.

Methotrexate, on the other hand, suppressed the B-cell compartment of the immune response. Although depression of antibody production was prolonged in methotrexate treated animals, susceptibility to FDV infection gradually returned after catabolism of the drug. This suggested that methotrexate suppressed lymphocyte proliferation.

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