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The effect of cyclophosphamide on the immune system of the pig

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ABBREVIATIONS

CY	cyclophosphamide
FPK	fetal porcine kidney
HBSS	Hanks' balanced salt solution
PBS	phosphate-buffered saline
PPV	porcine parvovirus
RFC	rosette-forming cells
sIg	surface immunoglobulin
SRBC	sheep red blood cells

INTRODUCTION

Immune suppression is the inability to mount a normal immune response and can be artificially induced in several ways, which may be antigen-specific or nonspecific. Antigen-specific suppression can be caused by an excess of specific antibody or by paralysis induced by specific antigen. Nonspecific immune suppression can be brought about by the use of antilymphocyte or antiimmunoglobulin serum, cytotoxic drugs, adrenocortical hormones, X-irradiation or surgical removal of lymphoid organs (in particular, thymectomy). Depending on the method used, immune suppression may affect primarily antibody-mediated immunity or cell-mediated immunity or both. For example, surgical removal of the thymus affects mainly cell-mediated functions, whereas cytotoxic drugs have some effect on humoral and cellular immunity (Webb and Winkelstein, 1978).

The present study was designed to develop a means of causing immunosuppression in the pig for use in studies on the pathogenesis of infectious diseases in this species. A primary criterion in the selection of an agent was its ability to cause immune suppression without mortality. Thymectomy in the pig has been previously shown to suppress some *in vivo* and *in vitro* functions of cell-mediated immunity, without prolonging graft survival, and treatment

with antilymphocyte serum as well magnified the observed immune suppression (Binns et al., 1972). It was decided not to use these two methods in the present study because of the poor degree of immunosuppression obtained by these workers, the expense and time involved in surgical manipulations and the variability inherent in the use of antilymphocyte sera (Bach, 1975). The cytotoxic drug cyclophosphamide (CY) was selected because it causes reproducible and well-characterized immune suppression in many animal species (Bach, 1975).

Cyclophosphamide acts as an alkylating agent, causing damage to nucleic acids, including breaks in DNA strands, cross-links between DNA strands and cross-links between DNA and RNA or protein. Cyclophosphamide therefore prevents mitosis and kills cells undergoing multiplication and cells with a high rate of RNA or protein synthesis (Bach, 1975). The alkylating activity is not present in CY itself, but is dependent on the production of metabolites of CY by a microsomal enzyme system in the liver (Bach, 1975).

Cyclophosphamide appears to be selectively cytotoxic for lymphocytes at intermediate doses (Petrov et al., 1971), but it does have some effect on all rapidly dividing tissues, such as bone marrow, gastrointestinal tract and hair follicles (Bach, 1975).

In human medicine, CY is used as an immunosuppressant

in organ transplantation and treatment of immunologic disease (Webb and Winkelstein, 1978). Experimentally, CY has been used in numerous studies to elucidate some aspects of the immune response (for example Turk and Poulter, 1972b; Glaser, 1979; Ramshaw *et al.*, 1977) as well as in the investigation of the pathogenesis of various bacterial and viral infections (see Literature Review).

A preliminary study on the effect of CY in the pig suggested that in this species CY causes depletion of lymphoid tissues; however, no attempt was made to determine a dosage schedule which caused immune suppression without mortality, and no functional tests of the immune response were carried out (Anderson *et al.*, 1974). In the present study it was considered necessary to develop in the pig a nonlethal CY treatment regimen which produced maximal immune suppression with minimal effects on other tissues, and then to characterize further the effects of CY on the immune system of the pig.

LITERATURE REVIEW

Many techniques are available for investigating the immune response, and thus for characterizing the effects of immunosuppressive procedures. In this literature review, CY will be discussed in relation to its effect on some commonly investigated parameters.

Cyclophosphamide Dosage and Toxicity

A wide variety of doses of CY has been used to cause immune suppression. Cyclophosphamide may be administered as a high dose in a single injection, or as a lower dose repeated several times. Table 1 summarizes the treatment schedules used experimentally in different species by a number of workers. The route of administration is usually intraperitoneal but the intramuscular, subcutaneous, intravenous and oral routes have occasionally been used (see Table 1).

The dosages of CY which produce toxicity vary with animal species, sex and presence of normal bacterial flora. Kovacs and Steinberg (1972) described 3 toxic syndromes caused by CY in mice. Single high doses (600 mg/kg) caused rapid death which was not prevented by bone marrow transplantation. Lower single doses (400 mg/kg) caused leukopenia and death within 2 weeks and daily doses of 40 mg/kg

Table 1. Immunosuppressive doses of cyclophosphamide used in different species

Species	Dose of CY (mg/kg)	Route	Number of treatments	Frequency	Reference
Mouse	100	s.c.	1	-	Ramshaw et al. (1977)
	150	i.p.	1	-	Maguire et al. (1979)
	300	i.p.	1	-	Turk and Poulter (1972a)
	400	i.p.	1	-	Stockman et al. (1973)
	10	s.c.	28	daily	Renoux and Renoux (1980)
	300	i.p.	3	every 2 days	Turk and Poulter (1972a)
Guinea pig	150	i.p.	1	-	Winkelstein (1977)
	300	i.p.	1	-	Turk and Poulter (1972a)
	10	i.p.	7	daily	Turk (1964)
	20	i.p.	14	daily	Maguire and Steers (1963)
	50	i.p.	16	weekly	Revell (1978)
Rat	20	i.m.	5	daily	Santos and Owens (1966a)
	60	i.m.	2	daily	Chakhava and Ruban (1979)
	180	i.m.	2	daily	Chakhava and Ruban (1979)
Chicken	70	i.p.	4	daily	Sharma and Lee (1977)
Ox	5	i.v.	10	every 2 days	Corrier et al. (1979)
Sheep	15	i.v.	10	weekly	Jun and Johnson (1979)
Dog	3	oral	15	daily	Putnam et al. (1975)
Human	0.7	oral	30-750	daily	Winkelstein et al. (1972)
	7	i.v.	7	daily	Santos et al. (1970)
	25	i.v.	3	daily	Santos et al. (1970)

led to profound bone marrow depression, superinfection, wasting and death. The 2 latter syndromes could be prevented by bone marrow transplantation. Turk and Poulter (1972a) observed that three injections of 300 mg/kg at 48 hour intervals were "usually fatal" in mice. Dose-response curves for suppression of the antibody response and for mortality have been determined for CY in mice (Berenbaum and Brown, 1964). On the basis of these curves, Berenbaum and Brown calculated that the therapeutic index (the ratio of the 50% lethal dose to the dose which causes 50% suppression of the mean antibody response) was 11.0; a 50% lethal dose of 500 mg/kg was observed and the dose causing 50% antibody suppression was 45 mg/kg.

Cyclophosphamide causes toxicity at lower doses in rats than in mice. Göing _et al_. (1970) reported a 17% mortality rate at 160 mg/kg in rats. Chakhava and Ruban (1979) observed deaths in conventionally-raised rats, but not in gnotobiotic rats, treated with single doses of 160-180 mg/kg. Male mice appear to be more susceptible to the toxic effects of CY than female mice; 92% of males died after treatment with 400 mg/kg whereas 100% of females survived 500 mg/kg (Stockman _et al_. 1973).

There are no published data relating CY dosage to toxicity in pigs.

Effect of Cyclophosphamide on Cells Involved in the Immune Response

Circulating leukocytes

Although not strictly involved in the immune response, neutrophils will be considered here since they are important effectors of immune function. Cyclophosphamide causes depression of the total blood leukocyte count. Fried and Johnson (1968) reported a 67% depression of the total leukocyte count in CY-treated mice. This depression is contributed to by a decrease in both circulating neutrophils and lymphocytes. The suppressive effect of CY on these cell populations has been reported in mice, guinea pigs (Turk and Poulter, 1972b), rats (Göing *et al.*, 1970), chickens (Linna *et al.*, 1972), cattle (Wagner *et al.*, 1976), pigs (Anderson *et al.*, 1974) and humans (Winkelstein *et al.*, 1972).

Lymphoid tissues and lymphocyte subpopulations

Histopathological studies have demonstrated that CY depletes lymphocytes from lymphoid tissues, with a more dramatic effect on B cells than on T cells. Linna *et al.* (1972) showed that CY treatment of newly hatched chickens caused almost complete disappearance of lymphocytes from the bursa of Fabricius, and irreversible destruction of the normal bursal architecture; lymphoid follicles (B-dependent

areas) were persistently absent from the spleen and cecal tonsils. The cortex of the thymus was initially depleted of lymphocytes; however, thymic morphology was completely restored within 12 days of cessation of treatment. Because of these differential effects the authors describe CY treatment of newly hatched chickens as a method of "chemical bursectomy". Rouse and Szenberg (1974) obtained similar results in chickens. In these experiments bursal regeneration was, however, seen in some chickens and a transient depletion of lymphocytes in periarteriolar lymphoid sheaths (T-dependent areas) of the spleen was also described.

Cyclophosphamide treatment of mice resulted in the complete absence of lymphocytes in the lymphoid follicles of the lymph nodes and spleen. The lymphocytes in the paracortical areas of the lymph nodes, however, were partially but never completely depleted and a considerable number of lymphocytes always remained in the periarteriolar sheaths of the spleen (Turk and Poulter, 1972a). Similar changes were observed in guinea pigs by the same authors. Thus in this study CY caused the complete removal of lymphocytes from B-dependent areas and partial depletion of T-dependent areas in lymph nodes and spleen. In the same study CY caused a dramatic depletion of lymphocytes from the cortex of the thymus of both mice and guinea pigs, with a mild depletion from the medulla. Lymphocyte repopulation of

lymph nodes, spleen and thymus was evident in mice 7 days after CY treatment, however no lymph follicles were seen at this time (Turk and Poulter, 1972a). A similar pattern of lymphocyte depletion in the thymus and peripheral lymphoid tissues has been observed in cattle (Corrier *et al.*, 1979) and in a preliminary study in pigs (Anderson *et al.*, 1974).

Various other methods have been used to investigate the effect of CY on T and B cell populations in lymphoid tissues. Bach and Dardenne (1972) examined the effect of the administration of thymus and or bone marrow cells on the ability of CY-treated mice to form antibodies to sheep erythrocytes. Antibody formation was suppressed in mice which received CY and could only be reconstituted with the injection of both thymus and bone marrow cells. These results do not provide any evidence for a preferential effect on B cells but they do suggest that CY depletes both T and B cells in peripheral lymphoid tissues.

Poulter and Turk (1972) investigated the effect of CY on the percentage of lymphocytes carrying the θ-antigen (a T cell marker) in the lymph nodes and spleen of mice. It was observed that CY caused an increase in the proportion of T cells. This confirms the suggestion that CY can have a greater effect on B cells than T cells in peripheral lymphoid tissues.

The effect of single CY treatments on B and T lymphocytes in the spleen of the mouse was evaluated by examining the presence of surface-associated immunoglobulins (a B cell marker), the electrophoretic mobility and the responsiveness to T and B cell mitogens of spleen cell suspensions (Dumont, 1974). Both B and T cells were depleted but the B cell component was more dramatically affected and started to recover much later than the T cell component.

Winkelstein (1977) demonstrated a 60-70% depletion of both T and B cells (measured by rosetting with rabbit erythrocytes and by complement rosettes, respectively) in guinea pig lymph nodes. This is at variance with the observation of Turk and Poulter (1972a) that on histological examination B cell depletion in guinea pig lymph nodes was much greater than T cell depletion. Perhaps this can be explained by a difference in dose rates since Winkelstein used half the single dose used by Turk and Poulter.

The effect of CY on T and B lymphocyte counts in peripheral blood has been investigated, with apparently conflicting results. Revell (1978) reported that long-term CY treatment in guinea pigs caused a depletion of circulating B cells with no significant depletion of circulating T cells. Winkelstein (1977) showed, however, that a single CY treatment in guinea pigs depleted both T and B lymphocytes

by 60-70%, with a more rapid recovery of T cells than B cells. The difference in results could be due to the difference in dosage schedules. Revell did not carry out T and B cell determinations until 7 days after the last CY administration, whereas Winkelstein observed that normal T cell counts were restored by 8 days after treatment and B cell counts were depressed until 14 days after treatment. This probably accounts for the apparent lack of T cell depletion in Revell's study. Anderson et al. (1974) observed a decrease in B cell percentages and an increase in T cell percentages in peripheral blood of CY-treated pigs. These results are difficult to evaluate since absolute cell counts were not presented and the T cell marker used (rosette formation with untreated sheep erythrocytes) has since been shown to be inadequate for T cell enumeration in pigs (Binns, 1978).

Thus, it seems apparent that under most experimental conditions CY exerts a greater depressive effect on B lymphocyte populations than on T lymphocyte populations. Reasons for this selective effect have been speculated upon by several workers. Cyclophosphamide destroys cells undergoing DNA synthesis, and therefore has a preferential effect on rapidly proliferating tissues (Bach, 1975). Thus, Lagrange et al. (1974) and Turk and Poulter (1972b) have hypothesized that CY selectively destroys B cells because

they are short-lived and undergoing more rapid proliferation than the long-lived T lymphocytes. Indeed, Miller and Cole (1967) demonstrated by using tritiated thymidine labeling that in rat lymph nodes long-lived cells were resistant to long-term CY treatment. Also, Turk and Poulter (1972b) examined the effect of CY on mouse lymphoid tissues labelled with radioactive iododeoxyuridine (which labels dividing cells) and sodium chromate (which labels nondividing and dividing cells). CY depleted the rapidly dividing lymphocytes without affecting the nondividing population. Dumont (1974) proposed that T cells may have a greater capacity for repairing the DNA lesions induced by CY's alkylating activity: cells which could repair DNA between mitoses would be protected from the cytotoxic effects of CY and thus B cells would be more dramatically affected than T cells. Bach (1975) has suggested that CY has a greater effect on B cells because they have a higher metabolic activity than T cells and CY not only affects cells undergoing mitosis but also cells with a high rate of RNA or protein synthesis. It seems likely that all these suggested mechanisms may play a part in the differential effect of CY on B and T cell populations.

Macrophages

Conflicting results have been obtained on the effect of CY on macrophage populations. One day after the completion of a course of 8 daily CY injections, macrophage numbers in induced peritoneal exudates from guinea pigs were significantly depressed (Winkelstein, 1973). However, 18 days after receiving a single injection of CY, guinea pigs had normal peritoneal macrophage numbers (Jokipii et al., 1979). In the latter study a depression may have been seen if testing had been carried out earlier.

Effect of Cyclophosphamide on Functions of Antibody-Mediated Immunity

Antibody production

Cyclophosphamide has been shown to suppress antibody formation in mice (Berenbaum and Brown, 1964; Stockman et al., 1973), rats (Göing et al., 1970; Santos and Owens, 1966a), guinea pigs (Turk and Parker, 1973), chickens (Lerman and Weidanz, 1970; Linna et al., 1972), dogs (Putnam et al., 1975), cattle (Corrier et al., 1979) and humans (Santos et al., 1970). The suppression is seen as a depression of nonspecific serum immunoglobulins, both IgG and IgM (Lerman and Weidanz, 1970; Corrier et al., 1979) and also as a suppression of antibodies to specific antigens. Such antigens include sheep, horse and chicken erythrocytes (Santos and

Owens, 1966a; Rouse and Szenberg, 1974; Corrier et al., 1979), protein antigens such as ovalbumin, bovine serum albumin and keyhole limpet hemocyanin (Turk and Parker, 1973; Lerman and Weidanz, 1970; Stockman et al., 1973), bacterial antigens such as Salmonella and Brucella (Lerman and Weidanz, 1970; Linna et al., 1972) and tumor antigens (Phillips, 1970).

The dose of CY is important in determining whether suppression of antibody formation occurs, and the degree of suppression. Berenbaum and Brown (1964) determined a dose response curve for CY with respect to the formation of antibodies to Salmonella typhi H antigens in mice. The curve was exponential and single injections of 20 mg/kg administered 2 days after immunization had no effect on the antibody response whereas doses of 50 mg/kg and greater reduced the antibody titer to below the lowest measurable level. Kerckhaert et al. (1977) showed that doses of 100 mg/kg of CY were required to suppress formation of antibodies to sheep red blood cells (SRBC) in mice. In this case the drug was administered 8 hours before immunization; thus, the difference in dose effect in the two studies may result from the difference in timing; differences in the antigen and the genetic background of the mice may also account for the discrepancy. Seventy percent of chickens which received 3 daily doses of 100 mg/kg of CY showed a response to killed

Brucella abortus organisms, whereas no chickens which received 3 doses of 200 mg/kg responded (Rouse and Szenberg, 1974). It is interesting to note that in mice antibody suppression occurred at a dose well below that which causes death due to toxicity (Berenbaum and Brown, 1964), whereas in chickens antibody suppression only occurred with lethal doses (Rouse and Szenberg, 1974).

The suppressive effect of CY on antibody responses also depends on whether single or multiple dosage regimens are used and the timing of CY administration in relation to antigen administration. Single doses of 50 to 125 mg/kg in humans did not suppress the mean antibody titer to Vi antigen from *Escherichia coli*, however 7 daily doses of 7 mg/kg completely eliminated the response (Santos *et al.*, 1970). In both cases the antigen was administered 4 hours after the first dose of CY. In man the active metabolites of CY fall to relatively low levels in serum within 4 hours of administration of CY (Brock *et al.*, 1971). Thus, perhaps single high doses administered before antigen sensitization do not remain active for long enough to exert a significant effect on B cell differentiation and antibody formation, whereas the continual replacement of CY with daily doses may allow this. This suggestion is supported by the observation that in mice single nonlethal doses of CY, administered 2 days after antigen sensitization, can eliminate the anti-

body response (Berenbaum and Brown, 1964). Presumably at this time CY is able to destroy the rapidly dividing, sensitized B cell population. Santos *et al*. (1970) treated humans with a variety of multiple low dose (7 to 25 mg/kg) CY schedules and compared the effect of *Pasteurella tularensis* vaccine administered 3 days before the first drug dose with that of Vi antigen administered 4 hours after the last drug dose. Most patients responded well to Vi antigen but in all cases there was no detectable serum titre to *P. tularensis*. Thus, in humans, the response to antigens administered after cessation of CY treatment is poorly suppressed. In contrast, chickens treated with 3 daily doses of CY at hatching remained unresponsive to antigens administered at 6 weeks of age (Lerman and Weidanz, 1970). It must be noted that at least 50% mortality resulted from the CY doses used in this study.

Santos and Owens (1966a) examined the effect of immunization of rats with SRBC at various times in relation to a 5-day course of injections of CY. Antigen was administered 5 days or 2 days before commencement of CY treatment, or 2 days after completion of CY treatment. In all cases the formation of specific antibodies was suppressed, however the different schedules had different effects on specific IgM and IgG. Immunization 5 days before CY treatment caused a prolongation of IgM production and a premature drop in IgG.

When the antigen was administered 2 days before CY treatment, there was only a short IgM response with an absence of IgG. Immunization 2 days after cessation of CY treatment also resulted in a lack of IgG, but IgM production was delayed. Thus, if antigen is administered up to several days before CY treatment and in certain situations after CY treatment, antibody formation is suppressed. Antibody production to antigens administered on the same day as a single CY treatment are usually suppressed in mice (Lagrange et al., 1974; Frisch and Davies, 1966; Phillips, 1970). Immunization at some stage during a course of multiple CY administrations also has been commonly used to suppress the humoral immune response in experimental situations, for example in dogs (Putnam et al., 1975) and cattle (Corrier et al., 1979).

Cyclophosphamide has a differential effect on different antibody classes. This has been demonstrated by Santos and Owens (1966a) as described above. Phillips (1970) treated mice carrying transplantable tumors with CY and examined antibody production by the regional lymph nodes. Cyclophosphamide eliminated a normal IgG1 response and delayed the IgG2 and IgM responses by 6 days. The results of these two studies suggest that CY has a more marked effect on IgG than on IgM. Chickens treated with CY at hatching showed severe depletion of both IgG and IgM in serum until at least 11 weeks of age (Lerman and Weidanz, 1970); some

birds which survived toxic doses of CY had no detectable levels of either class of immunoglobulin.

Cyclophosphamide may completely eliminate the antibody response or may simply lower the titer or delay a normal response. Which of these possible outcomes occurs is dependent on timing and dosage. The timing effect was demonstrated by Göing _et al_. (1970). Rats treated with 2 CY injections 24 hours apart showed no detectable response to SRBC administered 3 days before or 4 days after the first CY injection whereas when the antigen was administered at various intervals up to 38 days after CY treatment, successive increases in the response were seen, although at no stage were the titers as high as in controls. The dose effect was demonstrated well in mice by the work of Berenbaum and Brown (1964). Intermediate Cy doses lowered the antibody titre to _S. typhi_ and higher doses completely eliminated the response. A single dose of 100 mg/kg of Cy in mice served only to delay a normal antibody response to SRBC; 5 days after immunization antibody levels were depressed but at 7 days they were normal (Kerckhaert _et al_., 1977).

Most of the studies which have been discussed here demonstrate the suppression of primary responses. Cyclophosphamide may be equally efficient at suppressing secondary responses. Linna _et al_. (1972) showed that CY

administered before primary immunization depressed antibody formation in response to primary and secondary stimulation in chickens. Putnam et al. (1975) showed that in dogs CY treatment commencing at the time of secondary immunization suppressed the secondary response.

Immunological tolerance

Several workers have described the induction of immunological tolerance by CY.

Frisch and Davies (1966) treated mice with CY 24 hours after immunization with human erythrocytes. The mice were unable to respond to a second dose of human erythrocytes administered at a time when they had recovered their normal responsiveness to SRBC. In this study the mice remained refractory to stimulation with human erythrocytes for 10 days after the primary stimulus. Guinea pigs treated with egg albumin during a 14-day course of CY were tolerant to this antigen at 5-1/2 weeks after primary immunization, which was the only time of testing (Maguire and Steers, 1963). Many and Schwartz (1970) showed that the repeated administration of antigen could lead to the maintenance of CY-induced tolerance for as long as 9 months in some mice. The likelihood that CY will induce tolerance is greater the higher the dose of CY (Frisch and Davies, 1966) and the higher the dose of antigen (Aisenberg, 1967).

Aisenberg (1967) suggested that CY-induced tolerance is caused by antigenic stimulation in the presence of CY-inhibited DNA synthesis and mitosis, leading to the elimination of the specific immunological clone. In contrast, Ramshaw *et al*. (1977) obtained results suggesting that the maintenance of the unresponsive state is due to the activation of suppressor T cells by CY.

Response to B cell mitogens

The ability of lymphocytes to undergo transformation in response to mitogenic substances is a commonly used test of the functional integrity of these cells. Lymphocyte transformation is usually measured by the uptake of tritiated thymidine by lymphocytes in culture.

Cyclophosphamide treatment of mice completely abolished the *in vitro* proliferative response of their spleen cells to bacterial lipopolysaccharide, which is a B cell mitogen (Dumont, 1974). Stockman *et al*. (1973) described pokeweed mitogen (PWM) as a B cell mitogen but it is now generally considered to be mitogenic for both T and B cells (Douglas, 1978). Thus, Stockman's results demonstrating that spleen cells from CY-treated mice were unresponsive to PWM are difficult to interpret. However, the fact that the response to the T cell mitogen phytohaemagglutinin recovered more quickly than the response to PWM, supports the authors'

conclusion that B cell function was suppressed.

Effect of Cyclophosphamide on Functions of Cell-Mediated Immunity

Delayed hypersensitivity

Contact sensitivity will be included in the discussion on delayed hypersensitivity. Some authors consider contact sensitivity to be a type of delayed hypersensitivity reaction (Fudenberg *et al*., 1978) whereas others consider the two reactions to be distinct (Jokipii *et al*., 1979).

Cyclophosphamide suppresses delayed hypersensitivity reactions under certain experimental conditions. When administered after sensitization, CY suppressed the formation of contact sensitivity to oxazolone and picryl chloride in guinea pigs (Turk and Stone, 1963; Turk, 1964). Cyclophosphamide administered simultaneously with tuberculin purified protein derivative (PPD) prevented the cutaneous response of guinea pigs (Winkelstein, 1973). Turk (1964) showed that the depression of contact reactivity in the guinea pig was partly but not entirely due to an anti-inflammatory action of CY. Turk and Stone (1963) associated the CY-induced blockage of contact sensitivity in the guinea pig with the inhibition of development of large pyroninophilic cells in local lymph nodes during the early phase of sensitization. In the light of more recently acquired

knowledge, the suppression of delayed hypersensitivity by CY can probably be attributed to the toxic effect of CY on replicating T cells after sensitization.

In contrast, the administration of CY before sensitization has been shown to prolong and intensify delayed hypersensitivity reactions (Turk et al., 1972). Turk et al. (1972) showed that in CY-treated guinea pigs enhanced contact sensitivity to dinitrofluorobenzene (DNFB) occurred in the presence of a suppressed antibody response to DNFB and in the presence of apparently normal T cell areas in local lymph nodes. The CY effect was partially reproduced by splenectomy. These authors concluded that CY blocks a B lymphocyte response which modulates the normal development of delayed hypersensitivity. Lagrange et al. (1974) showed that, with increasing doses of immunogen, the antibody response to SRBC in mice increased, whereas the delayed response decreased. Moreover, CY administered before sensitization enhanced the delayed response. Thus, these workers also suggested that CY eliminates a modulating B cell response.

B cell deficient mice produced a normal contact sensitivity reaction which could be enhanced by CY to the same degree as the reaction of normal mice (Maguire et al., 1979). This suggests that CY does not remove suppressor B cells. Cell transfer experiments on CY-treated mice sensitized with methylated human serum albumin provided evidence

that CY eliminates suppressor T cells which normally regulate delayed hypersensitivity (Mitsuoka et al., 1979). Mitsuoka et al. (1979) suggested that two T cell subpopulations, with different recovery rates after CY treatment, are involved in delayed hypersensitivity responses. They proposed that effector T cells recover more rapidly than suppressor T cells. Cyclophosphamide treatment before antigen sensitization could result in the presence of recovered effector cells and the absence of suppressors at the time of sensitization; thus an enhanced response would occur. With CY treatment after sensitization both T cell populations would be affected simultaneously and a reduced response would occur. This seems to be the most plausible explanation for enhancement of delayed reactions by CY, since the results of Maguire et al. (1979) and Mitsuoka et al. (1979) are more concrete than the circumstantial evidence used to support the argument for the removal of B cell mediated suppression.

Conflicting results have been obtained with sensitization to PPD. Cyclophosphamide pretreatment enhanced delayed hypersensitivity to PPD in cattle (Corrier et al., 1979) but depressed the reaction in guinea pigs (Jokipii et al., 1979). Since the treatment schedule used by Jokipii et al. was the same as that used by Turk et al. (1972) to enhance contact sensitivity in guinea pigs, no explanation for this

inconsistency can be provided.

Graft rejection

Cyclophosphamide treatment of skin graft recipients prolongs graft survival. Cyclophosphamide prolonged the survival of skin grafts in rats by several days. The effect was greater when grafts were between rats compatible at the Ag-B histocompatibility locus than when grafts were between incompatible rats (Silvers et al., 1967). In chickens, CY administered at the time of hatching prolonged the survival of allogenic skin grafts by more than 11 days. The CY effect was seen if grafts were applied at 1 week of age but not at 3 weeks (Rouse and Szenberg, 1974), and the dosage used was lethal for some birds.

Cyclophosphamide has a greater effect on the survival of renal allografts than skin allografts in rats and the effect is dependent on CY dosage. Single treatments or 3 weekly treatments commencing at the time of grafting usually only prolonged renal graft survival by a few days. However, 21 daily treatments commencing at the time of grafting prolonged graft survival by more than 150 days (Kawabe et al., 1972). In contrast, renal allograft survival was not prolonged in dogs which received daily treatments of 2 or 3 mg/kg of CY (Putnam et al., 1975). Perhaps a higher dose than this is required for graft survival in the dog.

Santos *et al*. (1970) described a CY treatment protocol which allowed the establishment of bone marrow grafts in humans. The protocol involved the induction of immunological tolerance to the donor antigens. Donor antigen in the form of whole blood was administered 24 hours before commencement of a 4-day course of CY; donor marrow was administered 24 hours after the final CY treatment. Although in this case graft survival may have been due to tolerance, it is thought that CY-induced prolongation of graft survival is usually due to a nonspecific suppression of cell-mediated immunity. For instance, in the study of Kawabe *et al*. (1972), rats with surviving renal allografts were probably not tolerant to donor antigens since they rejected normally donor skin which was applied 60 days after renal grafting.

Graft versus host reactions

Graft versus host reactions are suppressed by CY in rats. Santos and Owens (1966b) demonstrated that CY treatment of the recipients of transplanted spleen cells suppressed the graft versus host reaction. Peripheral blood from CY-treated chickens had a reduced ability to mount a graft versus host reaction in chicken embryos (Lerman and Weidanz, 1970; Rouse and Szenberg, 1974).

Tumor immunity

Cyclophosphamide may affect the ability of an animal to mount a cytotoxic response to tumors. Fass and Fefer (1972) inoculated mice with lymphoma cells and investigated the *in vitro* cytotoxicity of their spleen cells to the tumor cells. If the mice were treated with CY 1 day after lymphoma inoculation, the cytotoxic response was depressed, whereas CY administered 1 day prior to tumor inoculation enhanced the response. Cyclophosphamide treatment before tumor administration also enhanced the response of mouse spleen cells to transplantable SV40-induced sarcoma, as measured by a tumor cell neutralization assay (Glaser, 1979). The transfer of normal T cells to CY-treated mice abolished the CY-induced augmentation of immune responsiveness, therefore Glaser postulated the existence of CY-sensitive T cells which normally suppress effector T cells against SV40-induced sarcomas. These results are similar to those obtained by Mitsuoka *et al*. (1979) who provided evidence that another function of cell-mediated immunity, delayed hypersensitivity, is also modulated by a CY-sensitive suppressor T cell population.

Functional capacity of T lymphocytes

In vivo administration of CY induces a decrease in the *in vitro* response of the surviving spleen, lymph node or blood lymphocytes to the T cell mitogens phytohaemagglutinin

(PHA) and concanavalin A (con A). This effect has been shown in mice (Stockman et al., 1973; Renoux and Renoux, 1980), guinea pigs (Winkelstein, 1973), chickens (Sharma and Lee, 1977), sheep (Jun and Johnson, 1979) and humans (Winkelstein et al., 1972). Active CY metabolites from the serum of CY-treated guinea pigs have also been shown to suppress the PHA response when incubated with blood lymphocytes from normal guinea pigs (Balow et al., 1977). As discussed previously, the response to the B and T cell mitogen PWM was suppressed by CY for longer than the PHA response, suggesting a more prolonged effect on B cell function than on T cell function (Stockman et al., 1973). Winkelstein et al. (1972) attributed the decreased PHA responsiveness to a "functional sterilization" of lymphocytes, as manifested by chromosomal deletions and changes in the structural integrity of chromosomes in lymphocytes from CY-treated patients.

Elaboration of macrophage inhibition factor (MIF) by lymph node cells from CY-treated guinea pigs was depressed (Winkelstein, 1973). Normal guinea pig lymphocytes, incubated with active CY metabolites in serum from CY-treated guinea pigs, were also deficient in MIF production (Balow et al., 1977). Incubation of normal guinea pig lymphocytes with active CY metabolites caused a transient suppression of in vitro antibody-dependent and mitogen-induced cellular

cytotoxicity reactions against chicken erythrocytes (Balow *et al.*, 1977).

Thus, CY probably not only exerts its effects on the immune system by the depletion of lymphocyte populations, but also by the depression of the functional integrity of surviving lymphocytes.

Effect of Cyclophosphamide on the Pathogenesis of Bacterial and Viral Infections

Cyclophosphamide has been widely used to study the pathogenesis of infectious diseases. The pathogenic effects of infectious organisms are usually potentiated by CY.

Cyclophosphamide caused an increase in the mortality of mice due to infection with *Pseudomonas aeruginosa*, in association with a marked increase in bacterial numbers in the liver, spleen and kidneys of infected mice (Harada *et al.*, 1979). Passively transferred immunity to *Listeria monocytogenes*, as determined by suppression of bacterial growth in liver and spleen, was prevented by CY treatment of mice which were the recipients of spleen cells from immune mice (Tripathy and Mackaness, 1969).

Cyclophosphamide treatment often converts normally nonfatal virus infections to fatal infections. Cyclophosphamide increased the mortality of mice due to West Nile

virus (Nathanson and Cole, 1971) and Coxsackie B3 virus (Rager-Zisman and Allison, 1973) from 0% to 100%, and that due to systemic vaccinia infection (Worthington et al., 1972) from 10% to 94%. In all these instances CY increased the titer of viremia. Virus titers in many tissues of the Coxsackie virus infected mice and in the brains of the West Nile virus infected mice were also increased by CY. The CY-induced increase in mortality due to vaccinia and Coxsackie infection was associated with a depression of serum antibody levels to these viruses. Transfer of specific antisera, at the time when antibody responses became detectable in virus-infected control animals, protected CY-treated animals against mortality (Worthington et al., 1972; Rager-Zisman and Allison, 1973). The level of viremia in chronic lactic dehydrogenase virus infections of mice was increased by CY treatment, once again in association with depressed antibody levels (DuBuy et al., 1971). Pretreatment with CY prevented human diploid cell strain rabies vaccine from protecting against rabies infection in mice, in the presence of a normal delayed hypersensitivity response and a depressed antibody response (Turner, 1979). Thus, CY has been useful in investigating the role of antibody-mediated immunity in the control of several systemic virus infections.

Smida and Smidova (1978) investigated the effect of

CY in chickens infected with avian sarcoma virus. CY increased the mortality and incidence of progressive tumor growth. The authors considered these results to be evidence for depression of cell-mediated immunity by CY. Mayo et al. (1977) showed that latent murine cytomegalovirus infections could be reactivated by CY, suggesting an involvement of the immune system in latent cytomegalovirus infections.

Cyclophosphamide does not always exacerbate the effect of virus infections. Cyclophosphamide has been shown to prevent disease caused by visna virus in sheep (Nathanson et al., 1976) and lymphocytic choriomeningitis virus in mice (Cole et al., 1971) without preventing brain infection, thus helping to elucidate the immunopathologic nature of these two diseases.

It is apparent from this review of the literature that CY has a wide range of effects on the immune system. Cyclophosphamide depletes lymphocyte populations and depresses lymphocyte functions, with a greater effect on B cells than on T cells. This selective effect is probably due to different rates of metabolism, replication and DNA repair between B and T lymphocytes. Cyclophosphamide suppresses antibody-mediated immunity and may enhance or suppress cell-mediated immunity; enhancement of cell-mediated immunity is probably due to a lesser effect on suppressor T cells than

on other T cell subpopulations. Only a brief report has been published on the effect of CY in the pig (Anderson *et al.*, 1974); this work suggested that CY has an effect on porcine lymphocyte populations similar to that seen in other species, however no tests of immune function or determinations of dose effects were presented. The present study was undertaken in order to characterize more fully the effects of CY on the immune system of the pig.

MATERIALS AND METHODS

Animals

One-week-old gnotobiotic Yorkshire pigs, one-week-old naturally farrowed Yorkshire pigs, six-week-old naturally farrowed Yorkshire pigs, ten-week-old naturally farrowed Berkshire pigs and ten-week-old naturally farrowed Yorkshire pigs were used in different experiments, as noted in the Results section. The gnotobiotic pigs were maintained under sterile conditions in isolators for the duration of the experiment (Waxler *et al.*, 1966). Naturally farrowed pigs were raised conventionally and fed commercial rations. The great deal of variability in types of pigs used in different experiments was necessitated by the cost and availability of animals.

Antibiotic Treatment of Animals

In experiments 4 and 5 all treated and control pigs received 6 intramuscular injections of 200,000 units of penicillin G procaine and 0.25g dihydrostreptomycin[1] per 5 kg body weight. The injections were administered simultaneously with the first CY treatment and at 2-day intervals thereafter.

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[1]Med-Tech Inc., Elwood, Kansas.

Collection of Peripheral Blood

In experiments where naturally farrowed pigs were used, all bleeding and injection sites were shaved and swabbed with 2% tincture of iodine. Blood was collected from the cranial vena cava into evacuated glass tubes[1] containing EDTA (1.4 g/ml blood) for complete blood counts, evacuated tubes[1] containing heparin (14.3 units/ml blood) for T and B cell assays and evacuated serum separation tubes[1] for titration of antibodies to SRBC and porcine parvovirus (PPV).

Cyclophosphamide Treatment of Animals

Cyclophosphamide[2] was dissolved in distilled water at a concentration of 100 mg/ml and passed through a .22μ filter. All treatments were administered intraperitoneally at the doses indicated in the Results section.

Antigen Administration

Sheep blood was collected by sterile venipuncture into an equal volume of Alsever's solution. The SRBC were washed 3 times in sterile phosphate-buffered saline (PBS) by

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[1]Vacutainer, Becton-Dickinson, Rutherford, New Jersey.

[2]Polysciences Inc., Warrington, Pennsylvania.

centrifugation at 400 x g for 10 minutes. The buffy coat was aspirated and the SRBC made up to a 20% suspension in sterile PBS. Animals were immunized by intravenous injection of 1 ml/kg of SRBC suspension.

Inactivated PPV vaccine was prepared by a modification of the method of Mengeling et al. (1979). Primary monolayer cultures of fetal porcine kidney (FPK) cells were subcultured in 250 ml flasks at a ratio of 1:2 and inoculated simultaneously with about 10^8 cell culture infective $doses_{50}$ ($CCID_{50}$) of strain NADL-2 of PPV. After incubation for 3 days at 37°C the cultures were frozen and thawed 3 times. The virus was inactivated by continuous stirring of infective medium and cell debris with a 0.3% volume of acetylethyleneimine for 4 hours at 37°C. Aliquots were collected before and after inactivation to test for viral infectivity and hemagglutinating activity. The vaccine was dialyzed with Eagle's balanced salt solution to remove acetylethyleneimine, then tested for infectivity by titration of cytopathic effects in FPK monolayers in Leighton tubes. The vaccine contained 10^8 $CCID_{50}$ of PPV/0.2 ml before inactivation, and 0 $CCID_{50}$/0.2 ml after inactivation. Hemagglutinating activity was determined by a tube agglutination test using guinea pig erythrocytes (Mengeling, 1972); the vaccine contained 1,024 hemagglutinating units/ml before and after inactivation. Animals received a single intramuscular

injection of 5 ml of vaccine.

Blood Counts

Total leukocyte counts were determined using an electronic cell counter[1]. The accuracy of this method was investigated by obtaining 10 estimates of the total leukocyte count for a single blood sample. The range of 10 values was 8,145-8,358 leukocytes/cu mm, with a mean of 8,245 and standard deviation of 64.

Differential white blood cell counts were carried out on smears stained with a commercial differential stain[2]. The percentage of each cell type was calculated after counting 100 cells on each of 2 smears from each blood sample. The results obtained for estimates of the differential count from 10 smears of a single blood sample are presented in Table 2. The total lymphocyte, neutrophil and monocyte counts were obtained by multiplying the total leukocyte count by the relevant percentage value. An animal was considered to have leukopenia, neutropenia or lymphopenia if the relevant cell count was significantly ($p < .01$) lower than the control value, as determined by the Student's t test.

[1]Coulter Counter Model Z, Coulter Electronics Inc., Hialeah, Florida.

[2]Diff-Quik, Harleco, Gibbstown, New Jersey.

Table 2. Range, mean and standard deviation of ten estimates of the differential white blood cell count from a single blood sample

	Range	Mean	Standard deviation
% Segmented neutrophils	31-43	38.6	3.6
% Band neutrophils	0-2	0.7	0.8
% Lymphocytes	44-55	50.0	3.5
% Monocytes	3-6	4.2	1.2
% Eosinophils	4-9	6.1	1.8
% Basophils	0-2	0.4	0.7

Antibody Titration

Anti-SRBC antibodies in serum were measured by a microtiter hemagglutination technique similar to that described by Reyero _et al_. (1979). Sera were heated at 56°C for 30 minutes, then placed in a 96-well round-bottom microtiter plate[1]. Two-fold serial dilutions were made in PBS, using a microtiter dilution apparatus with 50 µl microdiluters[2]. To each well 50 µl of a 0.5% suspension of thrice-washed SRBC in PBS were added. The plates were

[1]Titertek, Linbro Scientific Co. Inc., Hamden, Conn.

[2]Cooke Engineering Co., Alexandria, Virginia.

incubated for 90 minutes at room temperature before reading. Antibody titers are expressed as the reciprocal of the final serum dilution that caused hemagglutination. Each test serum was titrated in duplicate. Positive and negative control sera of known titers were used each time the test was carried out. Titers of 32 and 0 were consistently obtained with 10 repeat titrations of the positive and negative controls, respectively.

Antibodies to PPV in serum were titrated by a hemagglutination inhibition technique, as described by Mengeling (1972). Heat-inactivated (56°C, 30 minutes) serum (0.5 ml) was adsorbed with 0.5 ml of a 50% suspension of guinea pig erythrocytes and 0.05 g of kaolin for 30 minutes at room temperature then centrifuged at 1,000 x g for 15 minutes. An initial 5-fold dilution and serial 2-fold dilutions of the supernatant serum were made in PBS. Each dilution of serum (0.25 ml) was mixed with 4 hemagglutinating units of PPV (0.25 ml) and incubated at room temperature for 30 minutes. An aliquot of 0.5 ml of 0.5% guinea pig erythrocytes was added to each virus-serum mixture. These mixtures were allowed to stand for 90 minutes then examined for inhibition of hemagglutination.

Isolation of Peripheral Blood Lymphocytes

Lymphocytes were isolated from peripheral blood according to the method described by Paul *et al.* (1979). Ten ml of heparinized blood was added to 20 ml of 0.85% NaCl. Fifteen ml of diluted blood was layered onto 4 ml of Ficoll-diatrizoate[1] (specific gravity 1.08) in a 1.6 x 15 cm glass tube. After centrifugation at 400 x g for 25 minutes, the lymphocyte-rich layer was withdrawn and placed in 40 ml Hanks' balanced salt solution (HBSS). The cells were washed 3 times in HBSS by centrifugation at 400 x g for 10 minutes, then resuspended in HBSS to a concentration of 10^7 cells/ml.

The leukocyte fraction obtained by this method is not composed entirely of lymphocytes, but contains a small percentage of contaminating cells (mostly monocytes with some polymorphonuclear cells). Estimates of 2-7% (Salmon, 1979) and 26% (Shimizu *et al.*, 1976) have been obtained for the percentage of contaminating cells, as determined by endogenous peroxidase staining. Also, a percentage of lymphocytes do not react with either the T or B cell marker and are considered to be "null lymphocytes". Salmon (1979) has obtained an estimate of 15% null lymphocytes, but this is

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[1] Ficoll-Paque, Pharmacia Fine Chemicals, Piscataway, New Jersey.

not corrected for contaminating cells.

Since the percentage of contaminating cells in the lymphocyte-rich fraction was not measured in this study, the percentage of null lymphocytes could not be calculated. Thus, in calculating absolute T and B cell numbers it was assumed that all the lymphocytes present in the lymphocyte-rich fraction reacted with either the T or B cell marker. For example, absolute T cells were calculated as:

$$\frac{\%\ \text{RFC}}{\%\ \text{RFC} + \%\ \text{sIg-bearing cells}} \times \text{absolute lymphocyte count}$$

where

RFC = rosette-forming cells (T cells)

sIg-bearing cells = surface immunoglobulin-bearing cells (B cells)

The absolute lymphocyte count was calculated from total and differential white blood cell counts.

Erythrocyte Rosette Assay

T cells were assayed by rosette formation with dextran-treated sheep erythrocytes by a method similar to that described by Binns (1978). An aliquot of 0.1 ml of lymphocyte suspension (1 x 10^6 cells) was added to 0.2 ml of 1% SRBC in 6% dextran (m.w. = 150,000)[1] in a 1 ml conical

[1] Dextraven 150, Fisons Lim., Holmes Chapel, Cheshire, U.K.

centrifuge tube, mixed and incubated at 37°C for 10 minutes. The suspension was then centrifuged at 200 x g for 5 minutes at room temperature and incubated for about 16 hours at 4°C. Cells were resuspended by gently rocking the tube, and a drop of suspension was placed on a slide under a coverslip. Two hundred lymphocytes were counted by light microscopy and the percentage of RFC (defined as bearing 3 or more adherent RBC) was calculated. Ten independent estimates of the percentage of RFC from a single blood sample had a range of 59-67, a mean of 63 and a standard deviation of 2.9. Rosette-forming cells are illustrated in Figure 1.

Quantitation of Immunoglobulin-Bearing Cells

Surface membrane immunoglobulin-bearing lymphocytes (B cells) were identified by the method described by Paul _et al._ (1979). An aliquot of 0.1 ml of lymphocyte suspension (1 x 10^6 cells) was placed in a 1 ml conical centrifuge tube, which was then filled with PBS containing 0.2% sodium azide and 1% bovine serum albumin, and centrifuged at 800 x g for 2 minutes. The supernatant was removed and the cells were resuspended in 50 µl of a 1:10 dilution of fluorescein-conjugated rabbit anti-porcine IgG (heavy and light chain specificity),[1] then incubated at 4°C for 30 minutes. After

[1]Lot 10432, Cappel Laboratories Inc., Cochranville, Pennsylvania.

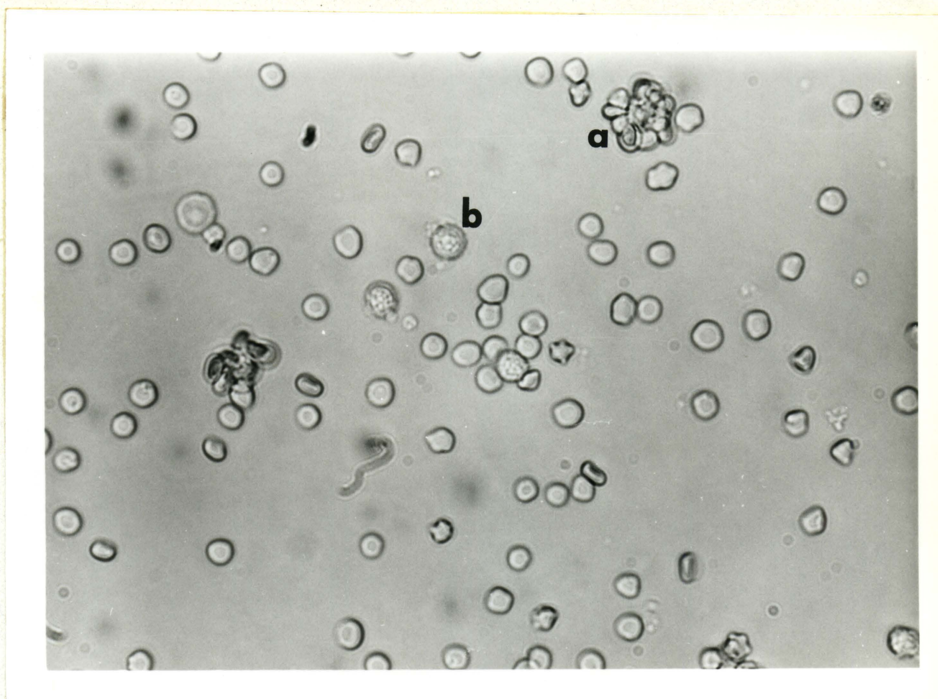


Figure 1. Rosette-forming cells in lymphocyte-rich fraction (x 500): (a) Rosette-forming cell, (b) Nonrosette-forming cell

incubation, the cells were washed 3 times with PBS containing sodium azide and bovine serum albumin, by centrifugation at 200 x g for 2 minutes. Cell suspensions were then examined by alternating phase and fluorescent microscopy. The percentage of cells positive for sIg was determined after counting 200 cells. Ten independent estimates of the percentage of sIg-bearing cells from a single blood sample had a range of 26-33, a mean of 30 and a standard deviation of 2.4.

Histopathology

Thymus, spleen and submandibular lymph nodes were fixed in formalin, sectioned and stained with hematoxylin and eosin using standard procedures. The sections were examined under light microscopy and various parameters quantitated. The concentrations of lymphocytes in the thymic medulla, lymphoid follicles and periarteriolar lymphoid sheath of the spleen, and the lymphoid follicles and paracortex of lymph nodes were calculated. Lymphocyte concentrations were assessed by randomly selecting 5 high-power fields from each section and counting the number of cells in a designated area of the field. The number of lymphoid follicles per low-power field of the spleen was calculated by taking the mean of the counts for 5 fields. The thickness of the cortex of the thymus was measured from

photomicrographs of 50 x magnification; 5 measurements for each animal were averaged. Thus the histological values obtained were not absolute counts, but purely for comparison of CY-treated and control tissues.

Statistical Analysis

The Student's t test was used to determine the statistical significance of differences between the means of treated and control groups of animals for leukocyte counts, T and B lymphocyte counts and histopathological data.

RESULTS

Determination of a Nonlethal Immunosuppressive Dose of Cyclophosphamide

Experiments 1 to 4 were carried out in order to determine a CY dosage schedule which caused immune suppression without mortality in the pig. Leukocyte counts were used as an easily measured indicator of CY's effect on the animal; it was assumed that CY could not cause immune suppression in the absence of lymphopenia. The antibody response to specific antigens was determined in order to assess the effect of CY on the humoral immune system. Thus, the parameters examined in seeking an appropriate dosage regimen were leukocyte counts, antibody response to SRBC or PPV, and mortality. The results of Experiments 1 to 4, with respect to these parameters, are summarized in Table 3.

In Experiments 1 to 4 blood was collected for white cell counts and titration of antibodies to the relevant antigen on day 0 (the day of commencement of CY treatment) and every 2 days until the termination of the experiment.

Experiment 1

One-week-old gnotobiotic Yorkshire pigs were used. All CY treatments were administered as a single injection on day 0. Three pigs received 25 mg/kg of CY, 3 pigs received 50 mg/kg of CY, 3 pigs received 75 mg/kg of CY and 3

Table 3. Summary of results obtained in the determination of an immunosuppressive but nonlethal dose of CY

Experiment	Age (weeks)	Treatment (mg/kg CY)	Number of pigs	% Survival	Leukopenia	Suppression of antibody response
1	1	25	3	100	–	N.A.[a,b]
		50	3	100	+	N.A.[b]
		100	3	0	N.A.[c]	N.A.[b]
2	1	50	3	100	+	–
		75	3	67	+	+
		100	3	0	+	N.A.[c]
3	6	50 + 20 x 2[d]	5	100	+	+
		75	4	100	+	–
4	10	20 x 3[d]	4	100	+	+
		30 x 3[d]	8	100	+	+
		50 x 3[d]	4	25	+	N.A.[c]

[a]N.A. - not applicable.

[b]Neither control nor treated animals responded to antigen inoculation.

[c]These pigs did not survive long enough for the appropriate criteria to be determined.

[d]All multiple dosages were administered as 3 injections, each 2 days apart.

pigs served as untreated controls. All pigs were inoculated with SRBC on day 0. The experiment was terminated on day 16.

All the pigs treated with 25 mg/kg showed neutropenia on days 4 and 6, but there was no significant depression of lymphocyte or total leukocyte counts. The pigs which received 50 mg/kg had significantly depressed total leukocytes, neutrophils and lymphocytes on days 4, 6 and 8. All animals treated with 100 mg/kg of CY died on day 2. The effect of CY on the antibody response to SRBC could not be assessed since none of the animals, including controls, showed any response whatsoever.

Experiment 2

One-week-old naturally farrowed Yorkshire pigs were used. The different CY treatments were single injections of 50 mg/kg, 75 mg/kg and 100 mg/kg, administered on day 0. Three pigs were assigned to each CY treatment group, and 3 pigs were used as untreated controls. All animals were inoculated with SRBC on day 0, and white blood cell counts and antibody titers were followed until day 16.

The animals which were treated with 50 mg/kg and 75 mg/kg of CY all had depressed lymphocyte counts on days 2 and 4, and depressed total leukocyte and neutrophil counts on days 4 and 6. One of the animals treated with 75 mg/kg and all of the animals which received 100 mg/kg died within

4 days. Control animals and those treated with 50 mg/kg responded to SRBC on day 6. The animals treated with 75 mg/kg did not respond at all; however, the significance of this is questionable since the control titers only rose from <1 (no hemagglutination by undiluted serum) to 4.

Experiment 3

In Experiment 3, six-week-old naturally farrowed Yorkshire pigs were used. Since the control response to SRBC in the previous experiments was so poor, it was decided to use as the antigen inactivated PPV, which usually evokes a higher antibody titer (Mengeling et al., 1979). Thus PPV-free animals were used in this experiment. Also, since in Experiment 2 suppression of antibody formation was only obtained with a dose that killed one animal, it was decided to attempt a schedule of multiple nontoxic doses.

Five pigs received 50 mg/kg of CY on day 0 then 20 mg/kg on each of days 2 and 4. Four pigs received 75 mg/kg of CY on day 0 and there were 5 untreated controls. Inactivated PPV vaccine was administered to all animals on day 0. The experiment was terminated on day 14. No animals died in this experiment.

Lymphopenia and neutropenia were seen in both CY treatment groups on days 4 and 6. Control animals and animals treated with 75 mg/kg had responded to PPV vaccine

by day 6, whereas the animals treated with the multiple dosage did not respond until day 8. Peak titers of CY-treated animals were similar to those of controls. All the CY-treated animals appeared depressed until about day 10; they also developed subcutaneous abscesses and cellulitis in the region of bleeding and injection sites.

Experiment 4

Ten-week-old naturally farrowed Yorkshire pigs were used. Since no PPV-free pigs were available, the antibody response to SRBC was examined; it was thought that ten-week-old pigs would respond better to SRBC than the immunologically immature one-week-old pigs used in Experiments 1 and 2. Since the antibody response was only delayed for 2 days by the multiple CY administrations used in Experiment 3, it was felt that better immune suppression could probably be obtained. Thus, in Experiment 4, three different multiple dosage regimens were examined. In this experiment all the CY-treated and control pigs received broad-spectrum antibiotics to prevent the bacterial infections seen in the pigs in Experiment 3.

Four pigs received 3 injections of 20 mg/kg of CY, 8 pigs received 3 injections of 30 mg/kg of CY, 4 pigs received 3 injections of 50 mg/kg of CY and 4 pigs served as untreated controls. Cyclophosphamide injections were administered on days 0, 2 and 4. Sheep red blood cells were

administered on day 0. The experiment ended on day 10. The schedule involving repeated injections of 30 mg/kg of CY was selected as the one to be used in further experiments; therefore, the results for animals which received this treatment will be presented in detail.

All CY treatment regimens caused a transient depletion of total leukocytes, neutrophils and lymphocytes. In animals which received 30 mg/kg injections of CY the maximum depression of total leukocytes (Figure 2), neutrophils (Figure 3) and lymphocytes (Figure 4) was seen on days 4 and 6, at which point lymphocyte counts were lowered by more than 60% and neutrophils were almost absent from peripheral blood. Total leukocyte counts of these pigs were significantly ($p<0.01$) lower than those of controls on days 4, 6 and 8. Neutrophil counts were significantly depressed on days 4 and 6, and lymphocyte counts were significantly lowered on days 2, 4, 6 and 8. In animals treated with 20 mg/kg injections, the lymphocyte count was depressed by 33% on day 6 and neutrophils were depressed by 90% on days 4 and 6. Recovery of both cell types had occurred by day 8. Three of the 4 animals which received 50 mg/kg injections of CY died on days 4, 8 and 10. The antibody response to SRBC was suppressed in animals which received 20 mg/kg and 30 mg/kg injections of CY, but the suppression lasted longer in the 30 mg/kg group. Control animals had mounted a

Figure 2. Total peripheral blood leukocyte counts of controls and of animals which received three 30 mg/kg injections of CY (Experiment 4). Points represent mean ± standard deviation

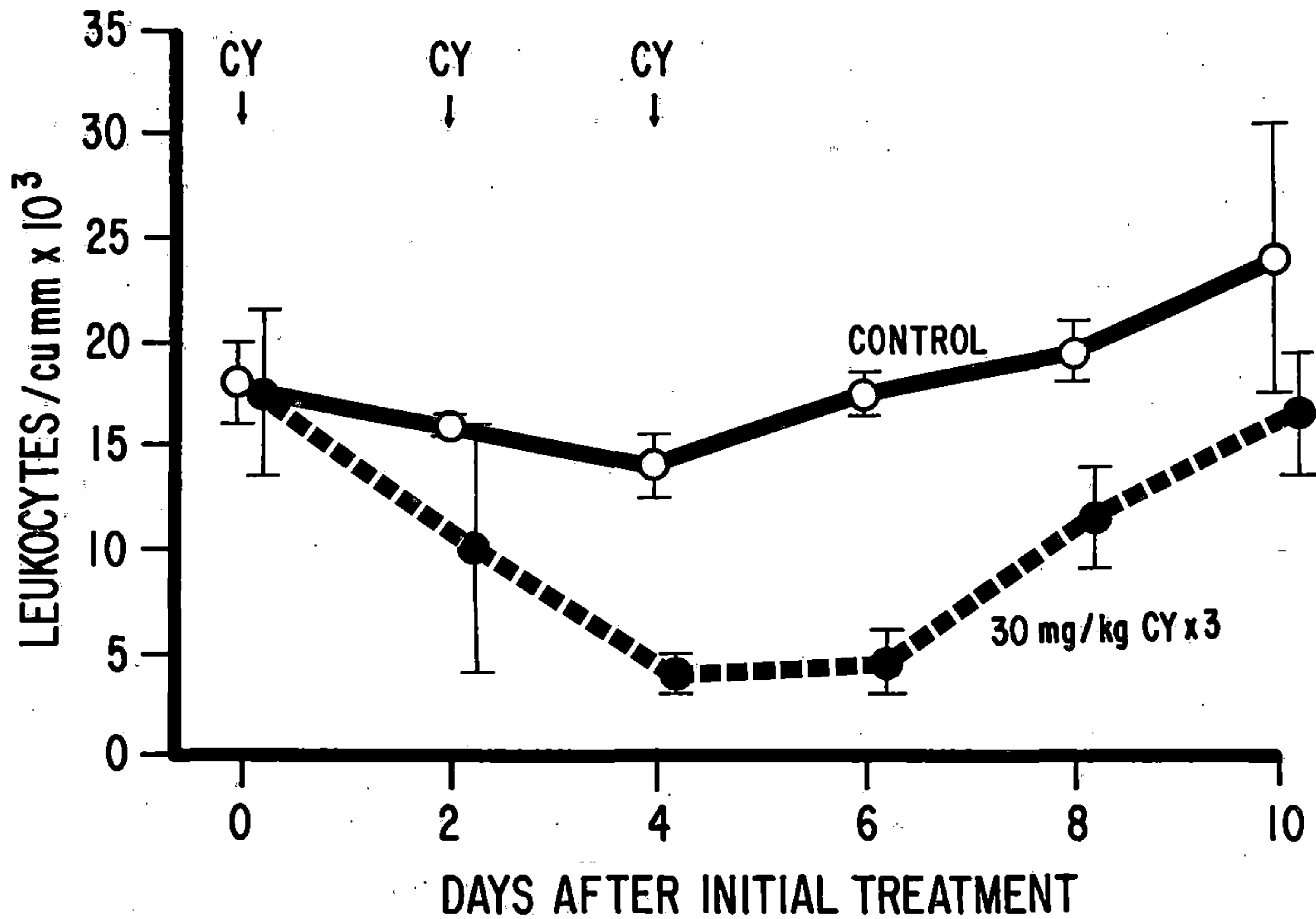

35
30
25
20
15
10
5
0
LEUKOCYTES/cu mm x 10^3
CY
CY
CY
CONTROL
30 mg/kg CY x 3
0
2
4
6
8
10
DAYS AFTER INITIAL TREATMENT

Figure 3. Peripheral blood neutrophil counts of controls and of animals which received three 30 mg/kg injections of CY (Experiment 4). Points represent mean ± standard deviation

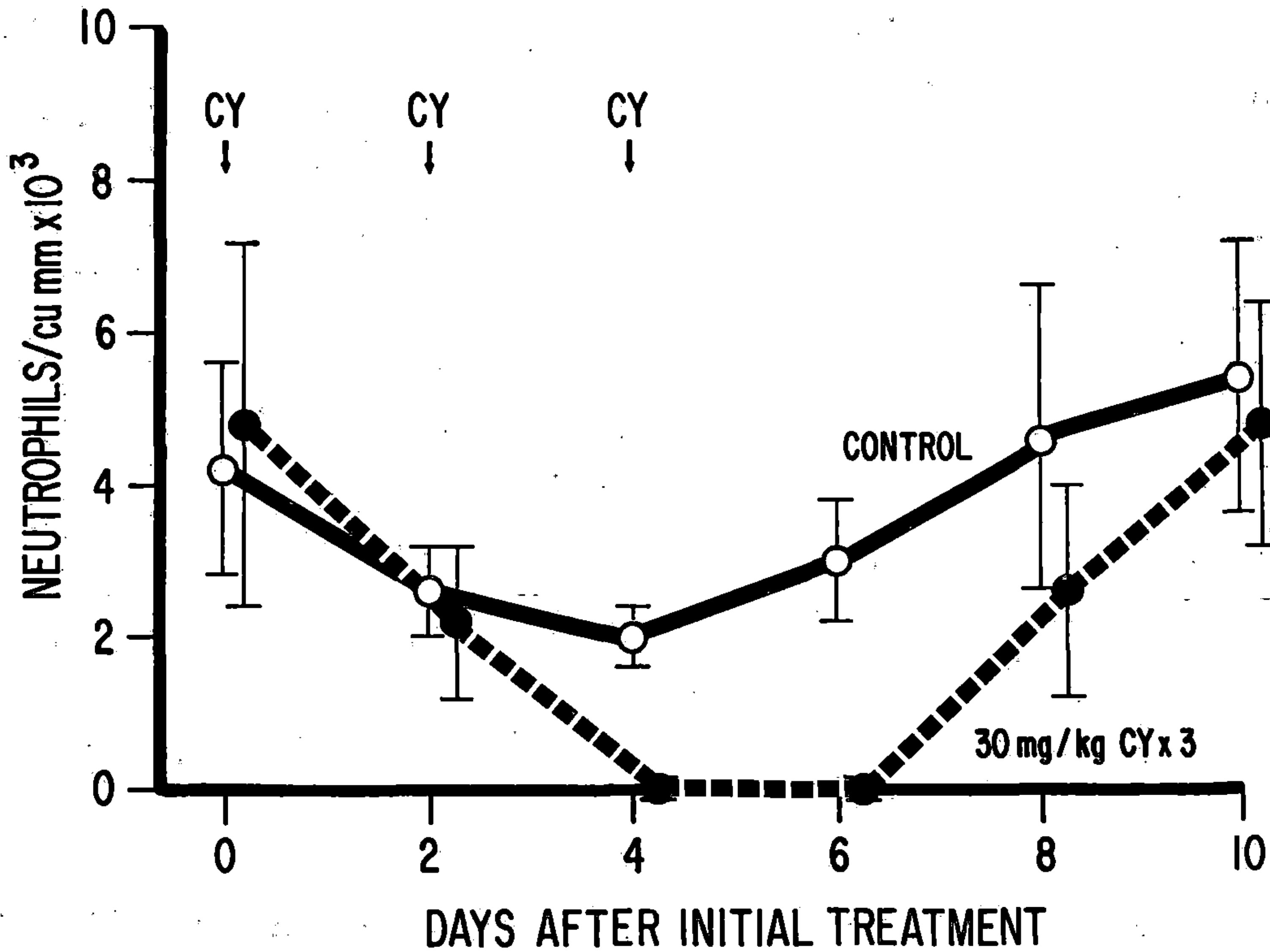
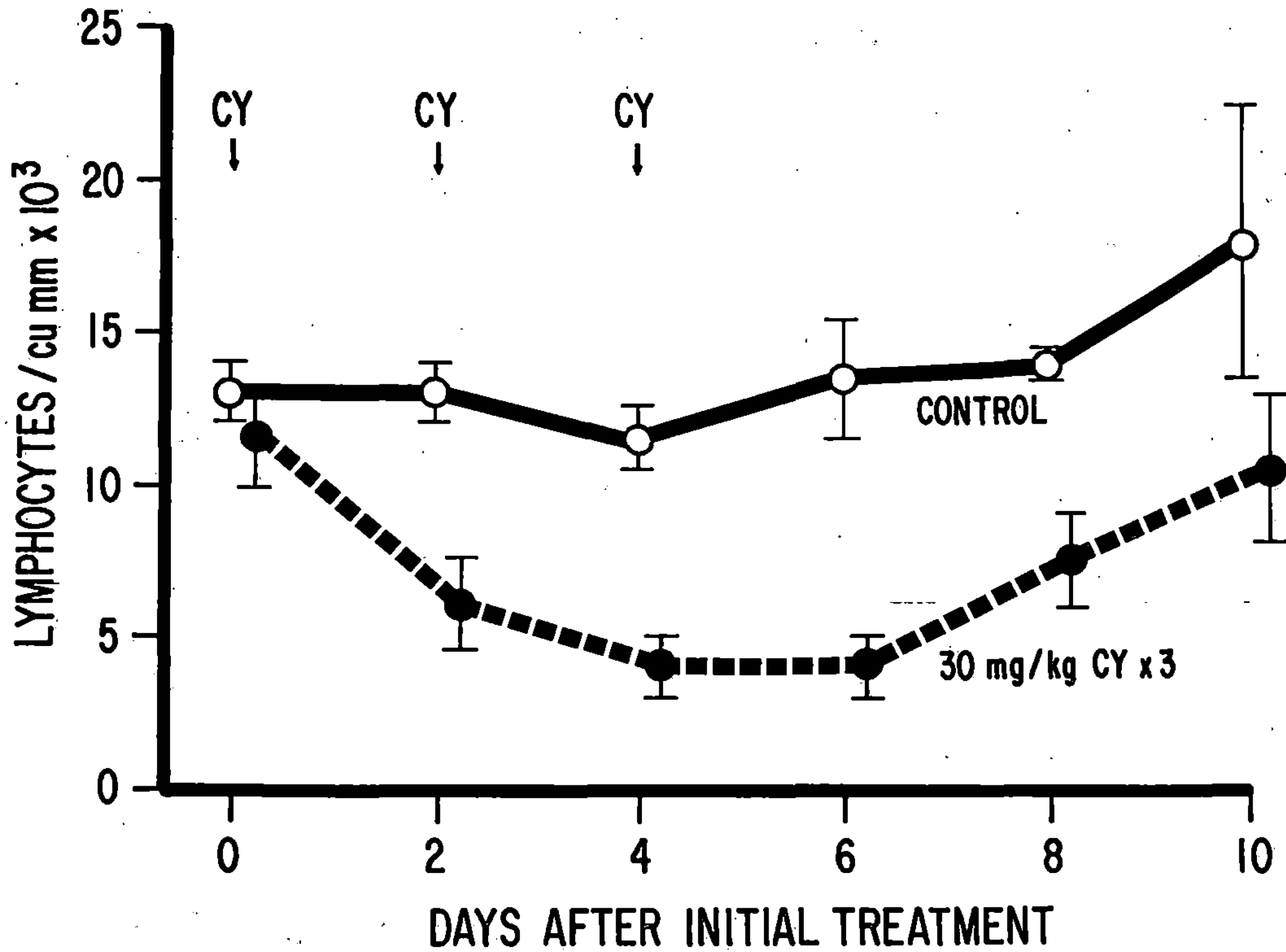

NEUTROPHILS/cu mm x 10^3
10
8
6
4
2
0
CY
CY
CY
CONTROL
30 mg/kg CY x 3
0
2
4
6
8
10
DAYS AFTER INITIAL TREATMENT

Figure 4. Peripheral blood lymphocyte counts of controls and of animals which received three 30 mg/kg injections of CY (Experiment 4). Points represent mean ± standard deviation



LYMPHOCYTES / cu mm x 10^3
25
20
15
10
5
0
CY
CY
CY
CONTROL
30 mg/kg CY x 3
0
2
4
6
8
10
DAYS AFTER INITIAL TREATMENT

detectable antibody response by 4 days after erythrocyte administration (Figure 5). Animals treated with 30 mg/kg injections of CY showed no detectable response until day 10 (6 days after the final CY treatment) and animals treated with 20 mg/kg injections had detectable titers on day 8. Some of the animals receiving the two lower dosages appeared depressed from day 1 until about day 8. No evidence of bacterial infections or adverse gastroenteric effects were seen.

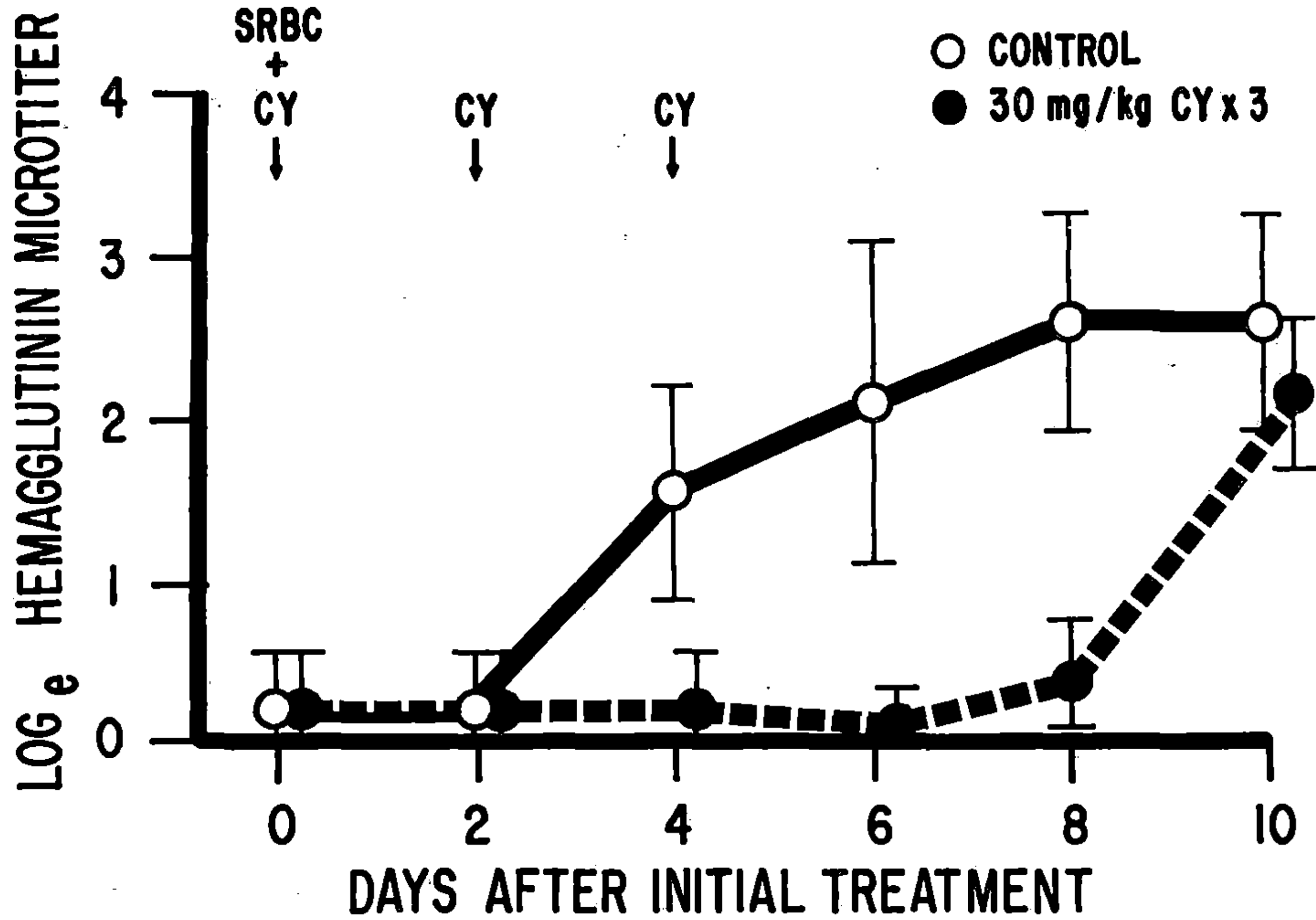
Further Investigation of the Effects of an Immunosuppressive Dose of Cyclophosphamide in the Pig - Experiment 5

Since in Experiment 4 the best immune suppression without mortality was obtained with repeated injections of 30 mg/kg of CY, this regimen was used in Experiment 5 to investigate further the effect of CY on the immune system of the pig. In this experiment one litter of ten-week-old Yorkshire pigs and two litters of ten-week-old Berkshire pigs were used. Equal numbers of pigs from each litter were assigned to each treatment.

Experimental design

Eight pigs received 3 CY treatments of 30 mg/kg on days 0, 2 and 4 (Treatment 1), 8 pigs received 6 CY treatments at 2-day intervals from day 0 (Treatment 2) and 8 pigs

Figure 5. Antibody response to sheep erythrocytes administered on day 0, in controls and in animals which received three 30 mg/kg injections of CY (Experiment 4). Points represent mean ± standard deviation of the $\log_e$ of the antibody titer


SRBC
+
CY
CY
CY
○ CONTROL
● 30 mg/kg CY x 3
LOG e HEMAGGLUTININ MICROTITER
0
1
2
3
4
0
2
4
6
8
10
DAYS AFTER INITIAL TREATMENT

served as untreated controls. At various intervals after the first treatment, total and differential leukocyte counts were carried out on peripheral blood. Two animals in Treatment 1 were killed on each of days 6, 8, and 13 and 2 animals in Treatment 2 were killed on each of days 13, 14, and 18. Two control animals were sacrificed on each of days 6, 13, and 18 and those killed on days 6 and 13 were used as controls for the CY-treated animals killed on days 8 and 14, respectively. The lymphoid tissues from the sacrificed animals were processed for histological examination. Two pigs from each treatment group, together with 2 controls, were inoculated with SRBC on day 0 in order to monitor the antibody response. T and B cell counts were not carried out on these animals, because it was thought that immunization with SRBC may have interfered with the results of the T cell rosette test.

Leukocyte counts

The leukocyte suppression in animals in Treatment 1 was similar to that seen in animals which received the same treatment in Experiment 4. Total leukocyte (Figure 6) and neutrophil (Figure 7) counts were significantly depressed on days 4, 6 and 8, whereas lymphocyte counts (Figure 8) were only significantly depleted on days 6 and 8. Recovery of all cell types was apparent on day 8. Total leukocyte (Figure 6), neutrophil (Figure 7) and lymphocyte

Figure 6. Total peripheral blood leukocyte counts of controls, of animals treated 3 times with CY (Experiment 5, Treatment 1) and of animals treated 6 times with CY (Experiment 5, Treatment 2). Points represent mean ± standard deviation

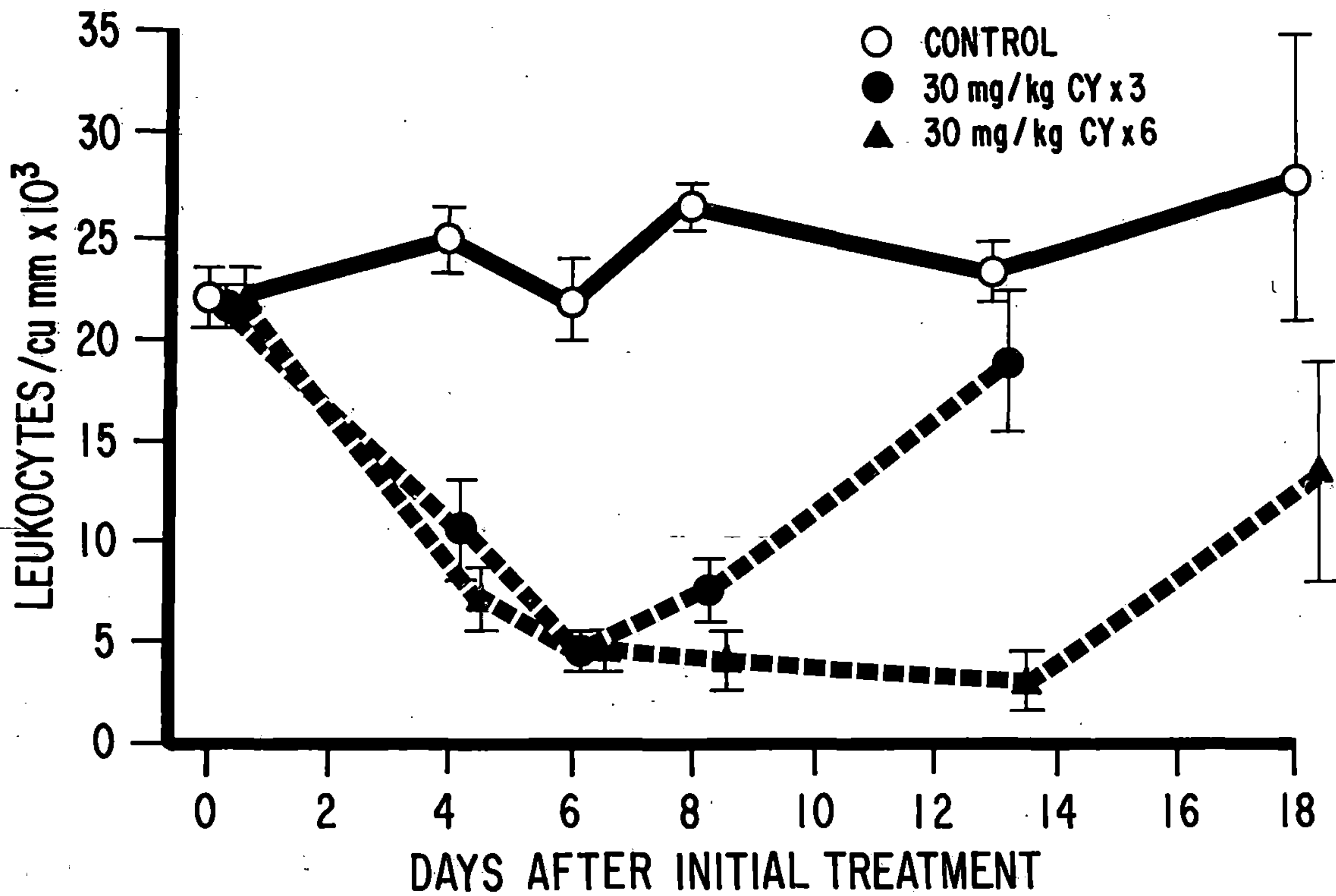

CONTROL
30 mg/kg CY x 3
30 mg/kg CY x 6
LEUKOCYTES /cu mm x 10^3
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
DAYS AFTER INITIAL TREATMENT

Figure 7. Peripheral blood neutrophil counts of controls, of animals treated 3 times with CY (Experiment 5, Treatment 1) and of animals treated 6 times with CY (Experiment 5, Treatment 2). Points represent mean ± standard deviation

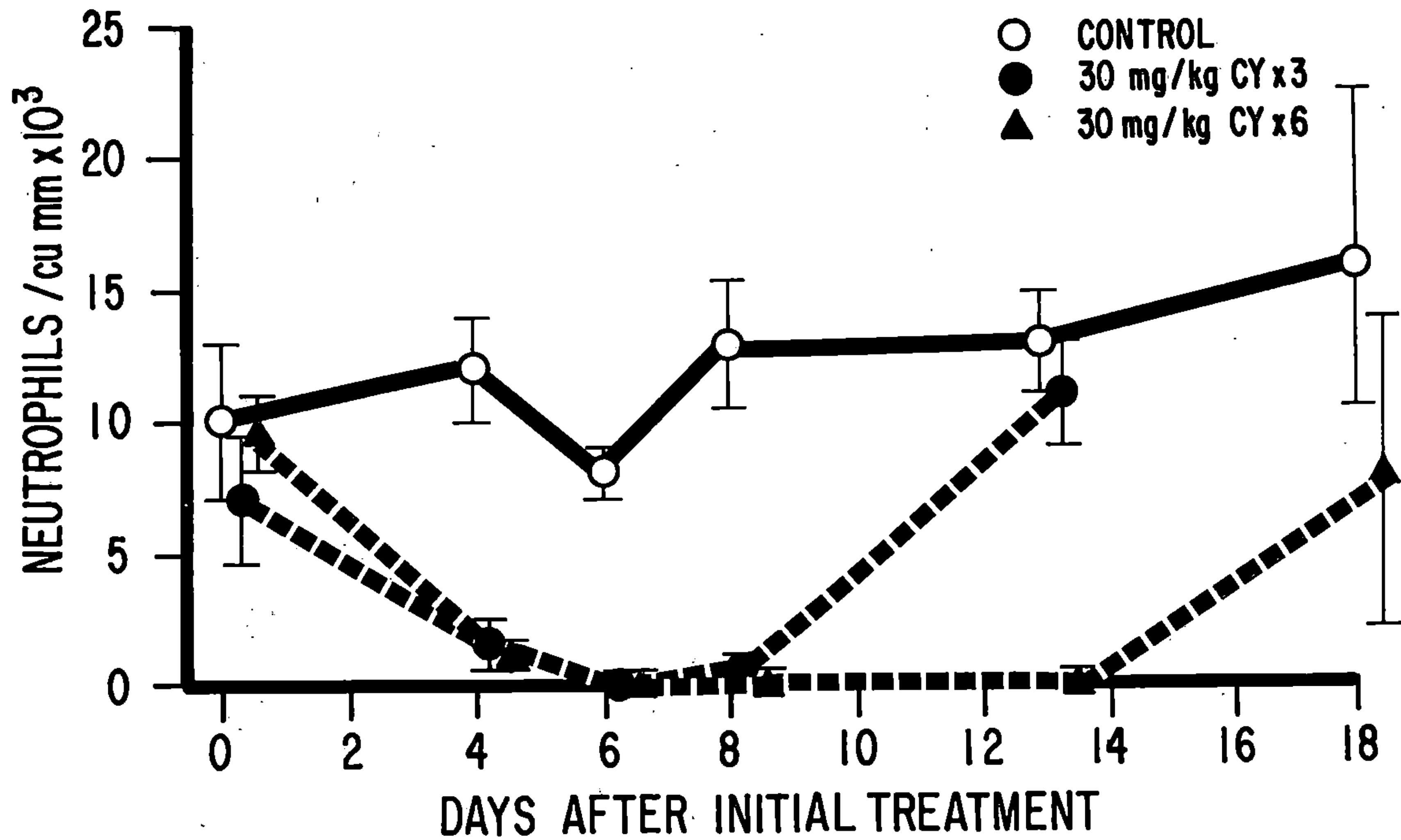
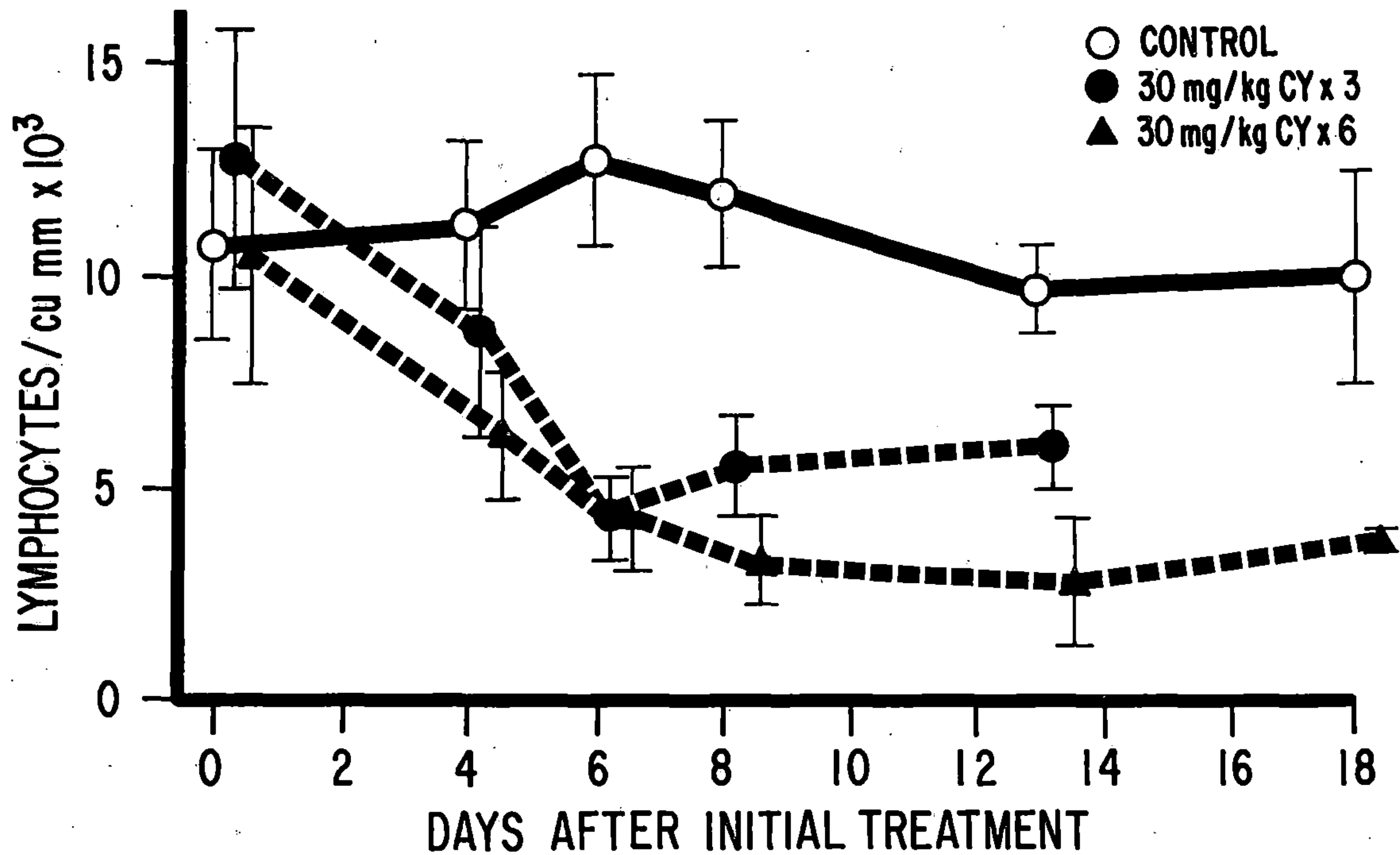

CONTROL
30 mg/kg CY x 3
30 mg/kg CY x 6
NEUTROPHILS /cu mm x 10^3
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
DAYS AFTER INITIAL TREATMENT

Figure 8. Peripheral blood lymphocyte counts of controls, of animals treated 3 times with CY (Experiment 5, Treatment 1) and of animals treated 6 times with CY (Experiment 5, Treatment 2). Points represent mean ± standard deviation


CONTROL
30 mg/kg CY x 3
30 mg/kg CY x 6
LYMPHOCYTES/cu mm x 10^3
0
5
10
15
0
2
4
6
8
10
12
14
16
18
DAYS AFTER INITIAL TREATMENT

counts (Figure 8) for the animals in Treatment 2 followed the same pattern as for those in Treatment 1 until day 6 and then continued to decrease slightly until day 13. Recovery of all cell types was evident on day 18, when counts were no longer significantly depressed. The numbers of monocytes in peripheral blood are shown in Table 4. Monocyte counts in CY-treated animals were depressed following CY treatment, however no significance can be attached to this because control counts showed an almost three-fold day to day variation.

T and B Lymphocytes in peripheral blood

The percentages of T lymphocytes in the lymphocyte-rich fraction were not affected by CY treatment (Table 5). However, CY caused a greater than 50% depletion of absolute T cell numbers (Figure 9). In animals which received Treatment 1, this depletion was only significant ($p<0.01$) on day 6, and a gradual increase in T cell numbers occurred after this point. In animals in Treatment 2, the depression of absolute T cell counts was significant from day 6 to day 13.

Cyclophosphamide caused a marked depression of both B cell percentages (Table 6) and absolute B cell counts (Figure 10). In the animals in Treatment 1, B cell percentages were significantly ($p<0.01$) lower than controls

Table 4. Monocyte counts in peripheral blood of CY-treated and control animals

Day	n	Control monocytes/ cu mm[a]	n	Treatment 1[b] monocytes/ cu mm	n	Treatment 2[c] monocytes/ cu mm
0	6	534 ± 187	4	796 ± 220	4	1,134 ± 475
4	6	996 ± 365	4	59 ± 55	4	122 ± 133
6	6	554 ± 237	4	286 ± 76	4	293 ± 174
8	4	995 ± 320	4	767 ± 484	4	319 ± 258
13	4	356 ± 101	2	788 ± 336	4	252 ± 168
18	2	659 ± 40	-	-	2	447 ± 173

[a]The values given represent the mean ± standard deviation of values from n animals.

[b]Animals which received 3 CY treatments (on days 0, 2 and 4).

[c]Animals which received 6 CY treatments (on days 0, 2, 4, 6, 8 and 10).

Table 5. Effect of CY on T cell percentages

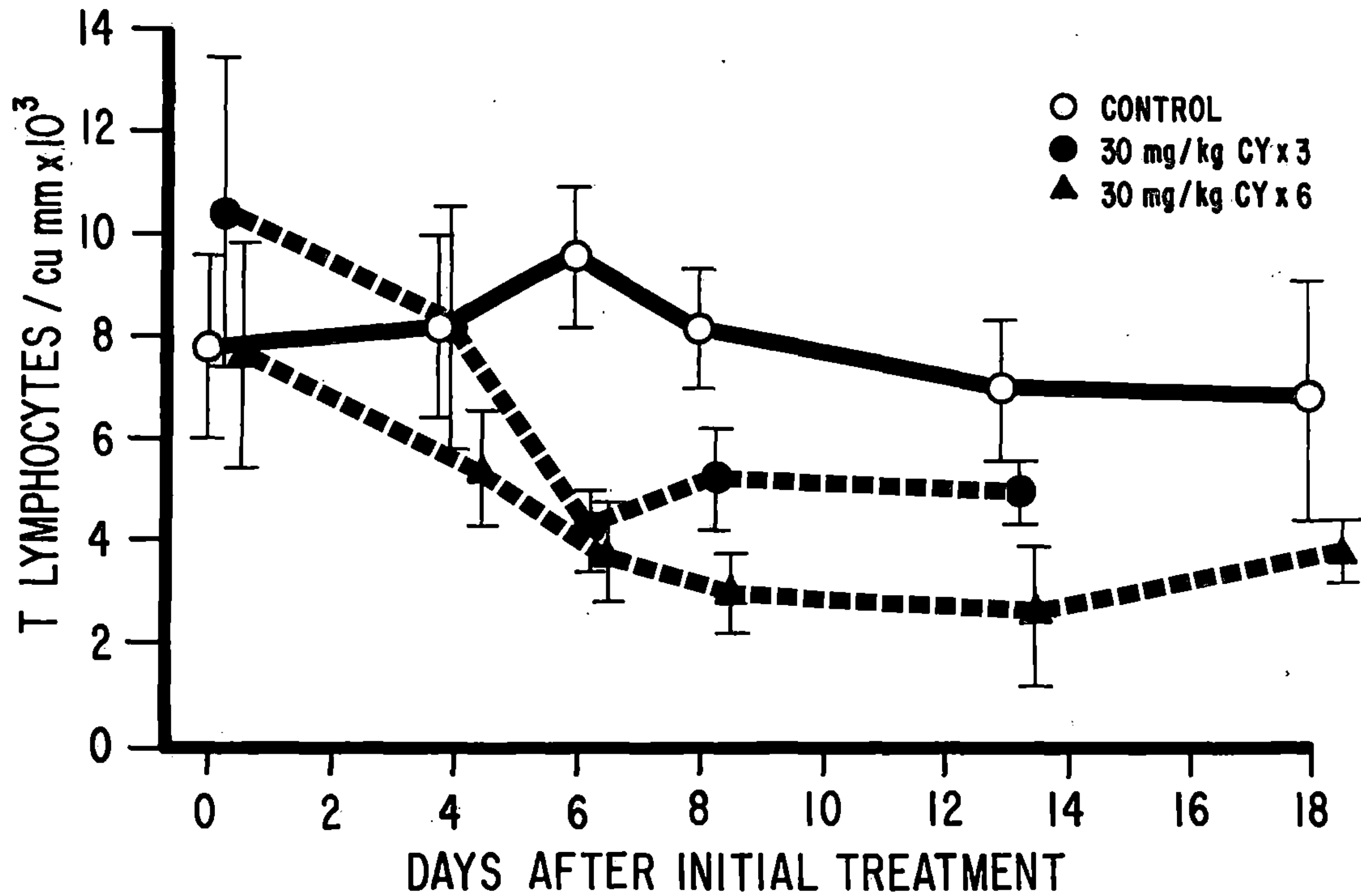
Day	n	Control % T cells[a]	n	Treatment 1[b] % T cells	n	Treatment 2[c] % T cells
0	6	62.1 ± 9.3	4	74.3 ± 5.3	4	65.8 ± 3.9
4	6	61.5 ± 6.2	4	70.8 ± 5.2	4	68.3 ± 6.6
6	6	60.5 ± 4.0	4	66.8 ± 9.9	4	65.0 ±13.1
8	4	54.0 ± 5.2	4	62.0 ± 9.7	4	66.0 ± 5.4
13	4	57.3 ± 6.1	2	57.5 ± 0.7	4	54.0 ± 1.9
18	2	53.0 ± 4.2	-	-	2	58.5 ± 2.1

[a]Percentage of T cells expressed as percentage of cells in the lymphocyte-rich fraction which form rosettes. The values given represent the mean ± standard deviation of values from n animals.

[b]Animals which received 3 CY treatments (on days 0, 2 and 4).

[c]Animals which received 6 CY treatments (on days 0, 2, 4, 6, 8 and 10).

Figure 9. Absolute T lymphocyte counts in peripheral blood of controls, of animals treated 3 times with CY (Treatment 1) and of animals treated 6 times with CY (Treatment 2). Points represent mean ± standard deviation



CONTROL
30 mg/kg CY x 3
30 mg/kg CY x 6
T LYMPHOCYTES / cu mm x 10^3
0
2
4
6
8
10
12
14
DAYS AFTER INITIAL TREATMENT
0
2
4
6
8
10
12
14
16
18

Table 6. Effect of CY on B cell percentages

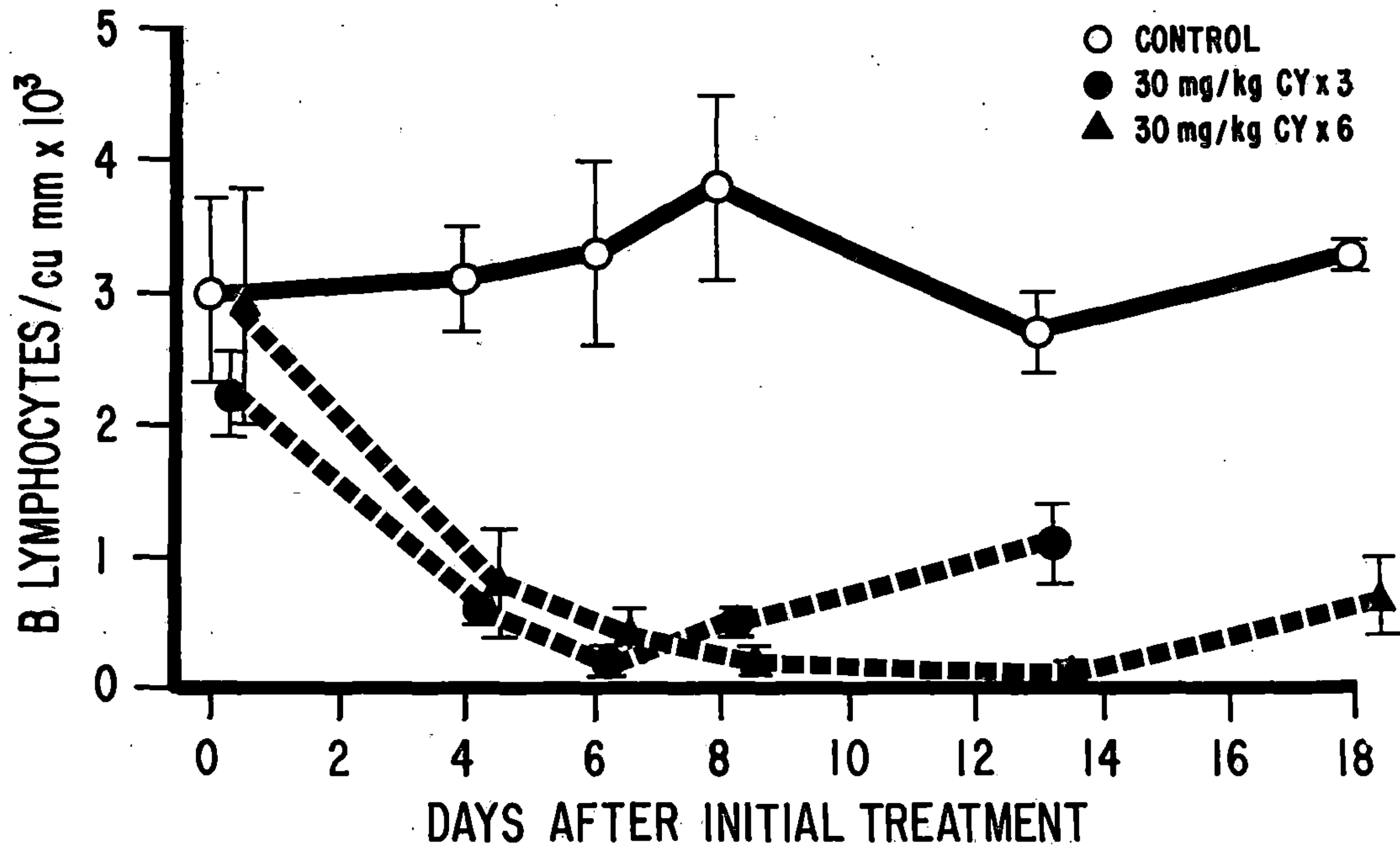
Day	n	Control % B cells[a]	n	Treatment 1[b] % B cells	n	Treatment 2[c] % B cells
0	6	24.2 ± 5.6	4	17.3 ± 6.7	4	24.5 ± 3.1
4	6	24.2 ± 6.2	4	5.5 ± 1.7	4	9.5 ± 4.7
6	6	21.0 ± 5.1	4	3.3 ± 1.3	4	5.3 ± 2.1
8	4	25.5 ± 6.1	4	5.3 ± 1.7	4	3.0 ± 2.4
13	4	23.0 ± 7.0	2	12.5 ± 3.5	4	1.8 ± 1.0
18	2	28.0 ± 11.3	-	-	2	12.0 ± 8.5

[a]Percentage of B cells expressed as percentage of cells in the lymphocyte-rich fraction which are sIg-positive. The values given represent the mean ± standard deviation of values from n animals.

[b]Animals which received 3 CY treatments (on days 0, 2 and 4).

[c]Animals which received 6 CY treatments (on days 0, 2, 4, 6, 8 and 10).

Figure 10. Absolute B lymphocyte counts in peripheral blood of controls, of animals treated 3 times with CY (Treatment 1) and of animals treated 6 times with CY (Treatment 2). Points represent mean ± standard deviation


CONTROL
30 mg/kg CY x 3
30 mg/kg CY x 6
B LYMPHOCYTES/cu mm x 10³
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16
18
DAYS AFTER INITIAL TREATMENT

from day 4 until day 8 (4 days after the last treatment). In this treatment group, absolute B cell counts were significantly below controls from day 2 to day 13 (9 days after the last CY treatment), although numbers had started to increase by day 8. The animals in Treatment 2 showed a similar response, with significant depression of B cell percentages until 3 days after the final treatment and depression of absolute B cell counts until 8 days after the final treatment. In both treatment groups there was a greater than 90% depletion of B lymphocytes at the point of maximum depression.

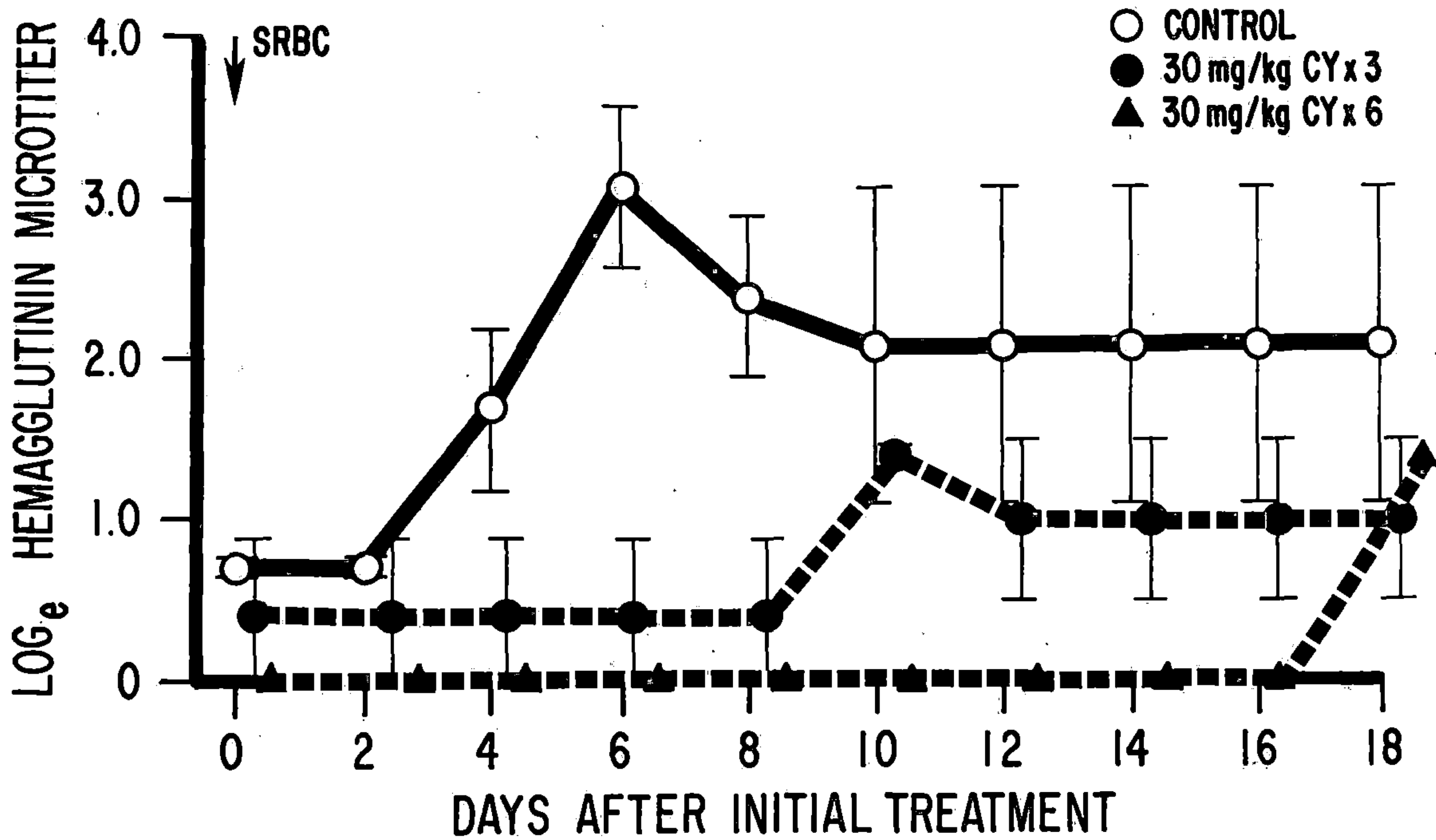
Antibody response to sheep erythrocytes

As in Experiment 4, control animals had detectable hemagglutinins in their serum by day 4 whereas animals in Treatment 1 had no detectable antibody titer until day 10 (Figure 11). The animals in Treatment 2 did not respond until day 18 (Figure 11).

Histological examination of lymphoid tissues

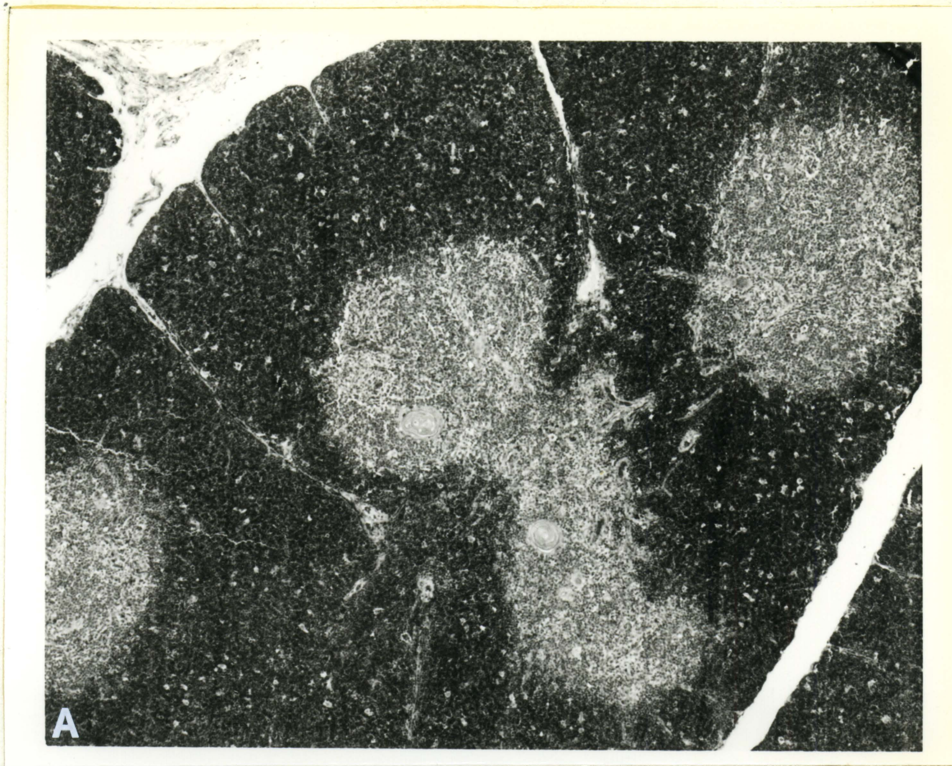
In the thymus of CY-treated animals there was a marked loss of distinction between the cortex and medulla, with thinning of the cortex due to lymphocyte depletion (Figures 12(a) and (b)). Table 7 shows the relative values for the thickness of the cortex in CY-treated and control animals. Cortical thickness was significantly reduced on day 6 (2 days

Figure 11. Antibody response to sheep erythrocytes administered on day 0, in controls, in animals treated 3 times with CY (Experiment 5, Treatment 1) and of animals treated 6 times with CY (Experiment 5, Treatment 2). Points represent mean ± standard deviation of the $\log_e$ of the antibody titer



LOG$_e$ HEMAGGLUTININ MICROTITER
4.0
3.0
2.0
1.0
0
SRBC
CONTROL
30 mg/kg CY x 3
30 mg/kg CY x 6
0
2
4
6
8
10
12
14
16
18
DAYS AFTER INITIAL TREATMENT

Figure 12. Effect of CY on morphology of the thymus. H & E stain; x 60: (a) Thymus of control pig sacrificed on day 13, (b) Thymus of a pig treated 6 times with CY (Treatment 2) and sacrificed on day 13, showing a depletion of cortical lymphocytes and loss of distinction between cortex and medulla


A

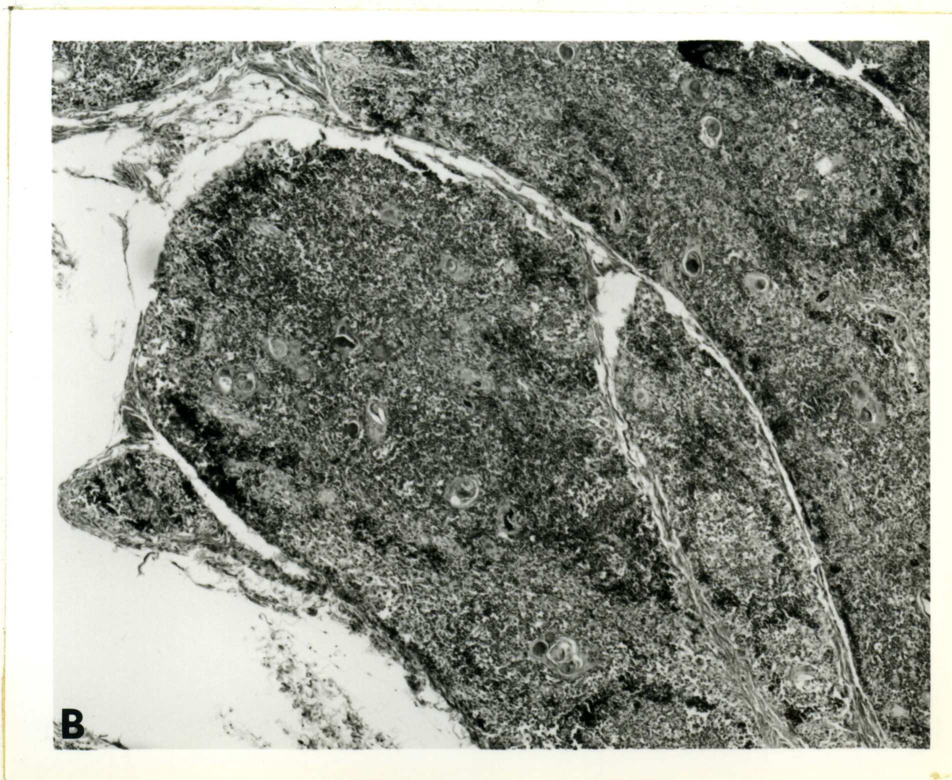

B

Table 7. Effect of CY on cortical thickness and medullary lymphocyte concentration in the thymus

Day killed	Control	Treatment 1[a]	Treatment 2[b]
Thickness of cortex[c,d]			
6	1.52 ± 0.37	0.34 ± 0.11*	
8		0.95 ± 0.07	
13	1.70 ± 0.16	1.15 ± 0.07	0.10 ± 0.07**
14			0.70 ± 0.42
18	1.53 ± 0.25		0.88 ± 1.17
Lymphocyte concentration in medulla[d,e]			
6	26.4 ± 1.1	21.1 ± 1.8	
8		20.8 ± 0.4	
13	23.9 ± 0.4	22.3 ± 3.2	13.7 ± 0.2**
14			18.8 ± 3.9
18	24.3 ± 2.5		19.0 ± 7.8

[a]Animals which received 3 CY treatments (on days 0, 2 and 4).

[b]Animals which received 6 CY treatments (on days 0, 2, 4, 6, 8 and 10).

[c]Cortical thickness was measured in centimeters from photomicrographs of 50X magnification, therefore the values given are relative.

[d]The values represent the mean ± standard deviation of values from 2 animals.

[e]Lymphocyte concentration expressed as the number of lymphocytes in a designated area of a high power field.

*Significantly different from control value ($p<0.05$).

**Significantly different from control value ($p<0.01$).

after the final CY treatment) in animals in Treatment 1 and on day 13 (3 days after the final treatment) in animals in Treatment 2. In both groups cortical lymphocyte repopulation had started to occur by 4 days after the final treatment, when values for cortical thickness were not significantly below control values. The concentration of lymphocytes in the medulla of the thymus was not affected by Treatment 1; it was significantly reduced on day 13 in the animals in Treatment 2, but recovery was evident on day 14 (Table 7). The standard deviations for cortical thickness and medullary lymphocyte concentration values of animals in Treatment 2 on day 18 are very large. This is because the thymus of one of the two animals killed on this day was still markedly atrophic whereas the thymus of the other was indistinguishable from normal.

Table 8 shows the changes in lymphoid areas of the spleen in CY-treated animals. Treatment 1 depleted the number of lymphocytes in lymphoid follicles on days 6 and 8 (Figures 13(a) and (b)) without significantly decreasing the number of follicles; there was no effect on lymphocyte concentration in the periarteriolar sheaths. In Treatment 2 depression of both follicle numbers and follicle lymphocyte concentrations was seen on days 13 and 14; values for day 18 were not significantly below controls. The concentration of lymphocytes in periarteriolar sheaths from animals

Table 8. Effect of CY on the number of splenic lymphoid follicles and lymphocyte concentration in lymphoid follicles and periarteriolar lymphoid sheaths in the spleen

Day killed	Control	Treatment 1[a]	Treatment 2[b]
Number of follicles[c,d]			
6	4.1 ± 1.3	1.2 ± 0.0	
8		1.0 ± 0.6	
13	4.9 ± 0.4	2.1 ± 0.4	0.2 ± 0.2**
14			0.7 ± 1.0*
18	4.0 ± 1.4		1.4 ± 2.0
Lymphocyte concentration in follicles[d,e]			
6	22.1 ± 2.1	9.4 ± 1.4*	
8		11.0 ± 1.4*	
13	21.8 ± 0.8	22.0 ± 0.7	3.2 ± 4.5*
14			10.5 ± 0.7**
18	23.0 ± 1.4		6.0 ± 8.5
Lymphocyte concentration in periarteriolar sheaths[d,e]			
6	23.6 ± 0.8	21.5 ± 1.5	
8		21.5 ± 2.1	
13	22.5 ± 0.7	23.3 ± 2.5	13.8 ± 0.6**
14			20.0 ± 2.1
18	25.3 ± 1.1		21.5 ± 0.7

[a]Animals which received 3 CY treatments (on days 0, 2 and 4).

[b]Animals which received 6 CY treatments (on days 0, 2, 4, 6, 8 and 10).

[c]Number of follicles per low power field.

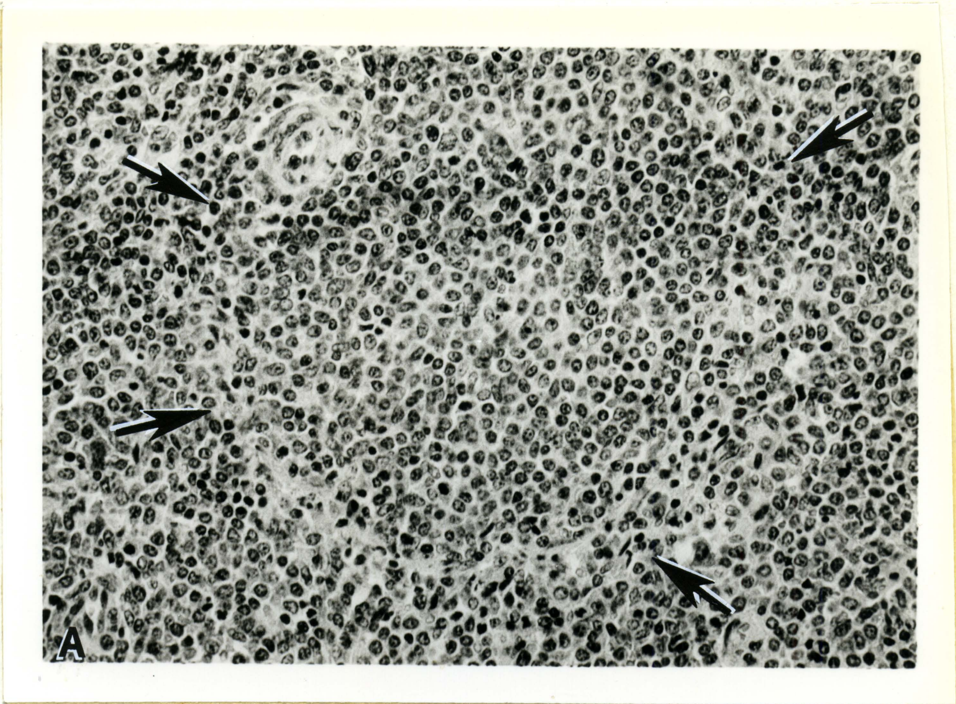
[d]The values represent the mean ± standard deviation of values from 2 animals.

[e]Lymphocyte concentration expressed as the number of lymphocytes in a designated area of a high power field.

*Significantly different from control value ($p<0.05$).

**Significantly different from control value ($p<0.01$).

Figure 13. Effect of CY on morphology of the spleen. H & E stain; x 260: (a) Spleen of control pig sacrificed on day 6, showing a lymphoid follicle (outlined by arrows), (b) Spleen of a pig treated 3 times with CY (Treatment 1) and sacrificed on day 6, showing depletion of lymphocytes from a lymphoid follicle (outlined by arrows)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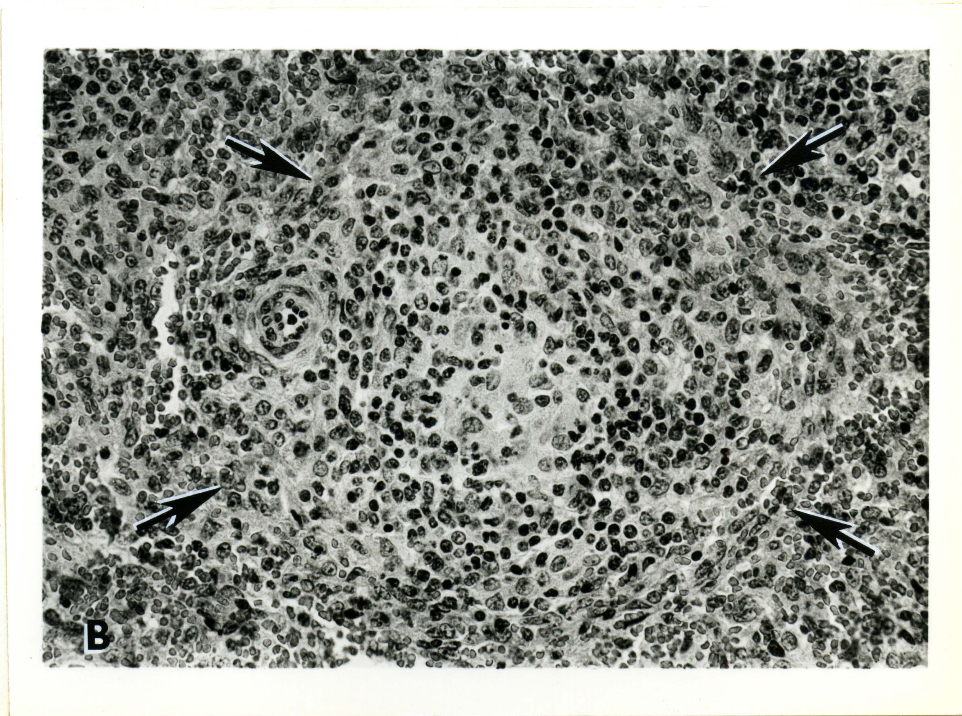

B

in Treatment 2 was depressed on day 13 but had already returned to normal by day 14.

CY-induced changes in the submandibular lymph nodes (Table 9) were less marked than in the spleen and thymus. Treatment 1 resulted in an initial depletion of lymphocytes in lymphoid follicles with recovery by day 13; no effect on the lymphocyte concentration in paracortical areas was seen. Treatment 2 caused a depression of the follicle lymphocyte concentration which was still apparent on day 18; paracortical areas were also depleted of lymphocytes but repopulation had occurred by day 14.

Table 9. Effect of CY on the concentration of lymphocytes in the lymphoid follicles and paracortical areas of the submandibular lymph node

Day killed	Control	Treatment 1[a]	Treatment 2[b]
Lymphocyte concentration in follicles[c,d]			
6	21.1 ± 0.4	10.5 ± 3.3*	
8		9.5 ± 0.7**	
13	22.2 ± 0.7	20.5 ± 0.3	5.3 ± 1.0**

[a]Animals which received 3 CY treatments (on days 0, 2 and 4).

[b]Animals which received 6 CY treatments (on days 0, 2, 4, 6, 8 and 10).

[c]Lymphocyte concentration expressed as the number of lymphocytes in a designated area of a high power field.

[d]The values represent the mean ± standard deviation of values from 2 animals.

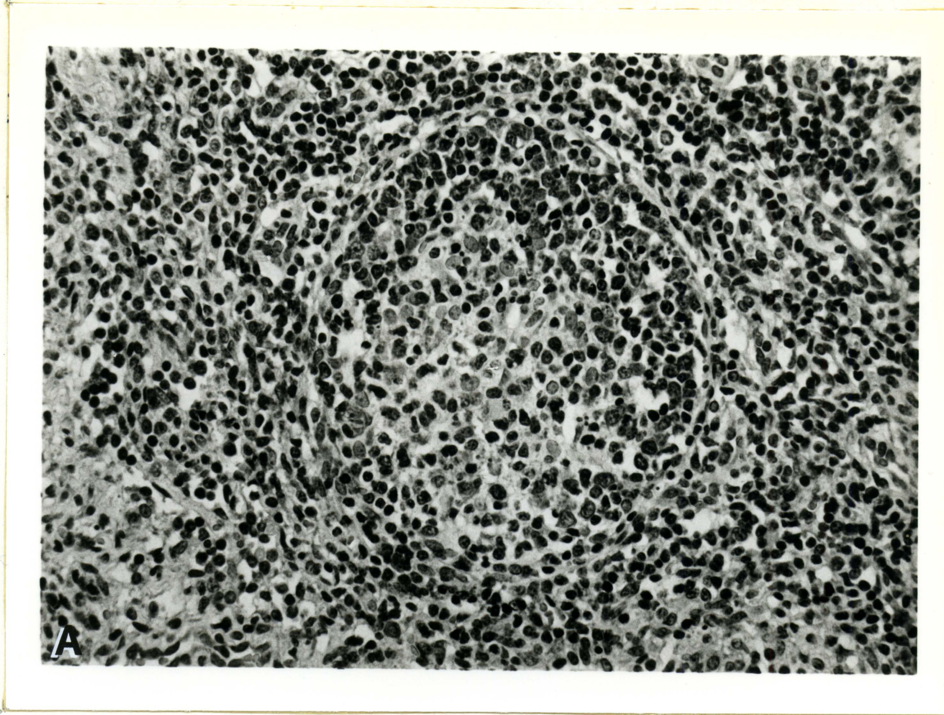
*Significantly different from control value ($p<0.05$).

**Significantly different from control value ($p<0.01$).

Table 9 (Continued)

Day killed	Control	Treatment 1	Treatment 2
Lymphocyte concentration in follicles (cont.)			
14			9.6 ± 0.7**
18	21.2 ± 0.5		13.4 ± 2.1*
Lymphocyte concentration in paracortical areas[c,d]			
6	29.5 ± 3.5	34.0 ± 1.4	
8		33.0 ± 2.1	
13	35.5 ± 0.7	30.1 ± 2.9	20.5 ± 0.7**
14			21.4 ± 4.6
18	30.2 ± 2.1		32.5 ± 3.4

Figure 14. Effect of CY on lymph node morphology. H & E stain; x 260: (a) Submandibular lymph node of control pig sacrificed on day 6, showing a lymphoid follicle, (b) Submandibular lymph node of a pig treated 3 times with CY (Treatment 1) and sacrificed on day 6, showing depletion of lymphocytes from the center of a lymphoid follicle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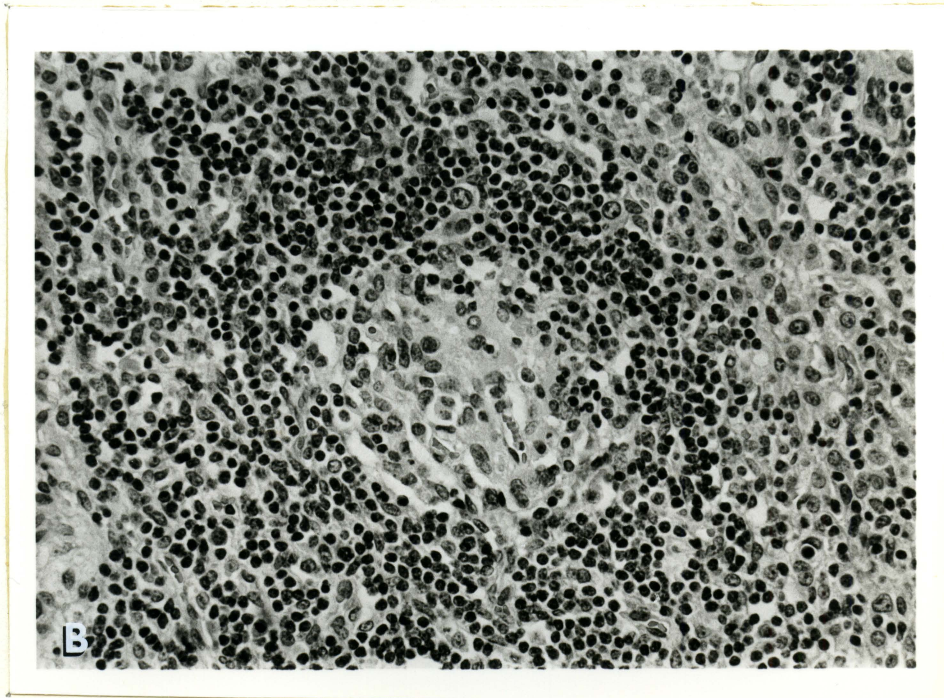
B

DISCUSSION

It was demonstrated in Experiments 1 and 2 that single injections of 100 mg/kg of CY cause 100% mortality within 4 days in one-week-old pigs. Anderson et al. (1974) also used single injections of 100 mg/kg in one-week-old pigs; in their paper no mention is made of mortality, however, all pigs were sacrificed 4 days after treatment. This dose is well below the dose of 600 mg/kg which Kovacs and Steinberg (1972) reported to cause rapid death in mice. Older pigs may be able to survive a higher dose than one-week-old pigs; in Experiment 4, however, 3 doses of 50 mg/kg administered every 2 days were lethal for ten-week-old pigs. Therefore this age group appears to be as susceptible as one-week-old pigs. The therapeutic index for single doses of CY in pigs could not be calculated because detailed dose-response experiments were not carried out; however, in Experiment 2 the antibody response was only suppressed at a dose which killed one of three pigs. This is different from the situation in mice, in which antibody formation was eliminated by 50 mg/kg of CY whereas toxicity only occurred at single doses above 200 mg/kg (Berenbaum and Brown, 1964). It is possible that lower single doses would have caused suppression of the antibody response in pigs if the antigen had been administered 2 days before CY, as in the work of

Berenbaum and Brown (1964).

A nonlethal multiple CY dosage regimen, in contrast, was shown to cause immune suppression in ten-week-old pigs. Multiple injections of 30 mg/kg, administered at two-day intervals, prevented the formation of detectable antibodies to sheep erythrocytes until several days after the last injection, whether 3 or 6 doses were used. Thus, the duration of immune suppression can be varied according to the number of injections used.

The depression of total blood leukocyte counts was in agreement with previous work in the pig (Anderson _et al_., 1974). The total leukocyte depletion was contributed to by the depletion of both lymphocytes and neutrophils. Cyclophosphamide also appeared to cause a depression of circulating monocyte counts; however, control values were too variable to place any significance on these counts. The variability was probably due to the low numbers of monocytes in blood smears. The intravascular half-life of neutrophils is approximately 6 hours, and the bone marrow is considered to contain a neutrophil reserve of approximately 4 to 8 days' supply (Schalm _et al_., 1975). Thus, the almost complete lack of circulating neutrophils within 4 days of initial treatment, and commencement of recovery within 4 days of cessation of treatment, can be explained as the prevention of mitotic activity of myeloblasts

by CY.

The kinetics of CY-induced depletion of lymphocytes in blood and lymphoid tissues are more difficult to explain. Cyclophosphamide could deplete lymphocytes by causing death or prevention of mitosis in lymphocytes, or sequestration of lymphocytes in nonlymphoid tissues. Turk and Poulter (1972b) showed that the depletion of lymphocytes in lymphoid tissues of mice was not due to localization in other organs, therefore it is unlikely that this mechanism plays a part in lymphocyte depletion in pigs. Turk and Poulter (1972a) attributed the effect of CY on murine lymphoid tissues to a purely mitostatic effect. The prevention of lymphocyte mitosis by CY is well-documented in mice (Turk and Poulter, 1972b) and calves (Wagner *et al.*, 1976) thus this mechanism is probably important in the present study. In contrast, Petrov *et al.* (1971) demonstrated that CY treatment of mice caused a greater suppression of graft versus host reactions than of hemopoiesis, and concluded that CY has a direct toxic effect on lymphocytes, as well as a mitostatic effect. Under the conditions of the present study it seems likely that CY has a lymphotoxic effect since in Experiment 4 there was a 60% depletion of circulating lymphocytes by 4 days after the initial treatment, and even the short-lived B lymphocytes are reported to have an average lifespan of 5 weeks (Sprent and Basten, 1973). This con-

clusion is supported by the data showing that circulating T cell numbers were depressed (Figure 9). The average life-span of circulating T lymphocytes is reportedly 4 to 6 months (Sprent and Basten, 1973), therefore a 50% depletion within 6 days of commencement of treatment must be partly due to a direct toxic effect on these cells. Thus, in the present study CY probably caused both death and mitostasis in lymphocytes.

Recovery of lymphocyte numbers in blood and lymphoid tissues was apparent within 4 days of cessation of CY administration in the animals which received Treatment 1. This rapid recovery could be explained by cyclophosphamide's mitostatic effect; however, it has been argued that CY also has a lymphotoxic effect in the present study. This lymphotoxic effect must mainly involve a mature, nondividing cell population, since if CY destroyed a significant proportion of the dividing precursor population, a much slower recovery would be expected.

As discussed in Materials and Methods, the lymphocyte-rich fraction used in this study to determine T and B cell numbers contains a certain percentage of contaminating cells. It seems likely that in the present study this percentage is relatively low since in controls the percentages of lymphocytes reacting with T cell and B cell markers usually added up to about 85%. This figure is similar to that

obtained by Salmon (1979), who estimated that 2 to 7% of the lymphocyte-rich fraction from pigs were contaminating cells, the remainder being null lymphocytes, whereas in another study in pigs, 26% of cells were contaminants and only 56% of cells reacted with the T and B cell markers (Shimizu et al., 1976).

Since CY treatment caused a decrease in the percentage of sIg-positive cells, without affecting the percentage of RFC, either the percentage of null lymphocytes or the percentage of contaminating nonlymphoid cells increased. It is not known which cell percentage increased since the percentage of contaminating cells was not determined. Also, total monocyte counts in controls were too variable to say whether monocyte numbers were affected by CY treatment, and thus whether they could have contributed to an increase in the percentage of cells not reacting with markers. It seems quite possible that the percentage of null lymphocytes increased, since Binns et al. (1977) demonstrated the existence in porcine blood of a cell population which neither formed rosettes with dextran-treated SRBC nor possessed sIg, and was depleted by thymectomy. Perhaps CY spares this thymus-dependent null cell population.

The percentage of null lymphocytes was not known, thus it was assumed in calculating absolute T and B cell counts that all lymphocytes were RFC or sIg-positive. The absolute cell counts were not entirely accurate but this method of

calculation has the effect of underestimating rather than overestimating the CY-induced depression of absolute counts. For example, T cell counts in Treatment 2 were depressed from $\frac{65.8}{65.8 + 24.5} \times 11{,}000 = 8{,}016$ on day 0 to $\frac{66.0}{66.0 + 3.0} \times 4{,}000 = 3{,}826$ on day 8 (i.e., 52% depletion), whereas if it had been assumed that all the cells which were not RFC or sIg-positive were null lymphocytes, the depression would have been from $\frac{65.8}{100} \times 11{,}000 = 7{,}238$ to $\frac{66.0}{100} \times 4{,}000 = 2{,}640$ (i.e., 64% depletion) (see formula for absolute T cell counts in Materials and Methods). Despite these limitations, the T and B cell counts do provide some useful information. B cells were depleted by CY more than T cells, since B cell percentages were depressed and T cell percentages were not. Cyclophosphamide treatment did cause a depletion of circulating T cells, although the absolute counts presented in this study probably underestimate the true magnitude of the depletion, and T cell counts returned to normal more rapidly than B cell counts.

In contrast to the results of the present study, Anderson et al. (1974) showed that CY treatment of one-week-old pigs decreased circulating B cell percentages and increased circulating T cell percentages without affecting absolute T cell numbers. Several factors may account for

this difference in results. These workers used a single high CY dose. Also, they only carried out T cell counts 4 days after CY treatment, that is, at a time when in the present study absolute T cell counts were not yet significantly depressed. The T cell marker used by Anderson *et al*. was rosette formation with untreated SRBC, but Binns (1978) subsequently showed that the percentage of porcine lymphocytes which formed rosettes was much higher with dextran-treated SRBC than with untreated SRBC. Perhaps CY depletes a subpopulation of T cells which forms rosettes with dextran-treated SRBC but not untreated SRBC, and spares a subpopulation which reacts with both. Thus, in the study of Anderson *et al*., absolute T cell counts of controls would not have included this CY-sensitive T cell subpopulation, and this would account for a lack of T cell depletion in CY-treated pigs. Evidence for the existence of a CY-insensitive T cell subpopulation has been produced by several other workers. Dumont (1974) showed that CY depleted only one of two murine T cell subpopulations which differed on the basis of electrophoretic mobility. Neta *et al*. (1977) showed that guinea pig thymus cells suppressed *in vitro* lymphoproliferative responses and that thymus cells from CY-treated guinea pigs had an enhanced suppressive ability. The authors suggested that thymic suppressor cells are resistant to CY treatment and that their activity becomes more pronounced

with the depletion of other types of thymic cells. Glaser (1979) and Mitsuoka *et al.* (1979) attributed the CY-induced enhancement of tumor immunity in mice and delayed hypersensitivity in mice, respectively, to the elimination of suppressor T cells and the sparing of effector T cells. Thus probably at least one T cell subpopulation is resistant to CY treatment.

The depletion of lymphocytes in lymphoid tissues by CY followed a pattern in the present study similar to that previously reported in pigs (Anderson *et al.*, 1974), as well as in guinea pigs, mice (Turk and Poulter, 1972a) and chickens (Lerman and Weidanz, 1970). The quantitative pathology carried out in the present study extends the descriptive work in pigs presented by Anderson *et al.* (1974) and confirmed that B cells are affected more than T cells. Three or 6 injections of CY depleted lymphocytes from the cortex of the thymus and 6 injections caused a depletion of medullary lymphocytes. Lymphocyte depletion from the B-dependent areas (lymphoid follicles) of the spleen lasted longer than the depletion from T-dependent areas (periarteriolar lymphoid sheaths). Similarly, depletion of lymphoid follicles in lymph nodes was more persistent and more marked than depletion of paracortical (T-dependent) areas. It is interesting to note that despite the "inverted" structure of the lymph node of the pig (McFarlin

and Binns, 1973), CY affects the B-dependent and T-dependent areas of pig lymph nodes in the same way as in other species.

The preferential effect of CY on B cells in peripheral blood and lymphoid tissues is probably partly due to the more rapid proliferation of B cells than of T cells, as suggested by Turk and Poulter (1972b). However, this may not be the sole reason since, as discussed previously, CY appears not only to affect dividing lymphocyte populations, but also nondividing lymphocytes. Thus, Bach's (1975) suggestion that the higher metabolic activity of B cells makes them more susceptible to the effect of CY is probably also important. T cell numbers probably return to normal more rapidly than B cell numbers because the B cell depletion is more dramatic than the T cell depletion.

In the present study, the formation of antibodies to SRBC was suppressed in CY-treated pigs. The period during which antibody formation was suppressed correlated well with the time of maximum depression of circulating B cells; in animals in Treatment 1 absolute B cell counts started to increase on day 8 and antibodies to SRBC were detectable in serum on day 10. Thus the suppression of the humoral immune response could have been solely due to a lack of cells able to differentiate into antibody-producing cells. Since SRBC is a T-dependent antigen (Douglas, 1978), however, the suppression of antibody formation could have been

partly due to the T cell depletion. Alternatively, since CY is known to suppress functions of T and B lymphocytes in other species, as witnessed by the depression of the *in vitro* lymphoblastogenic response to mitogens (Dumont, 1974; Stockman *et al.*, 1973), surviving lymphocytes may not be able to respond to antigen sensitization until after the CY-induced damage to DNA has been repaired. This latter suggestion may explain why in animals treated with 20 mg/kg injections of CY (Experiment 4), antibody formation was delayed, although lymphocyte counts were only depleted by 33%.

In summary, the effect of CY on the immune system of the pig was similar to that seen in other species with respect to the parameters observed in this investigation. Cyclophosphamide caused immune suppression, characterized by depression of the antibody response and depletion of blood and tissue lymphocyte populations, with a preferential effect on B cells, although T cells were significantly affected. The ability of CY administered in a multiple dosage regimen to cause reproducible immune suppression, followed by rapid recovery after cessation of treatment, should make CY a useful research tool in the pig.

Since in the present study CY caused a depletion of T cells, further investigations in this area could include examination of the effect of CY on *in vivo* and *in vitro*

functions of cell-mediated immunity in the pig, for instance delayed hypersensitivity and *in vitro* cytotoxicity by lymphocytes. Such experiments would aid in the use of CY to investigate the pathogenesis of infectious diseases in pigs.

SUMMARY

The effects of cyclophosphamide (CY) on circulating leukocytes, lymphoid tissues and the antibody response of the pig were examined. It was found that single nonlethal doses of CY caused leukopenia but did not suppress the antibody response to specific antigens. It was necessary to use a multiple dosage regimen to obtain immune suppression without mortality. Ten-week-old pigs were treated with intraperitoneal injections of 30 mg/kg CY at 2-day intervals. Three CY injections caused a depression of total leukocytes, neutrophils and lymphocytes. Circulating B lymphocyte percentages were depleted by 80% and absolute B lymphocyte counts by 90%. Circulating T lymphocyte percentages were not affected by CY treatment, but absolute T cell counts were depressed by 50%. Lymphocytes were depleted from the cortex of the thymus and from the spleen and lymph nodes with a preferential effect on B-dependent areas. All these variables were depressed for the duration of CY treatment and had started to return to normal by 4 days after the final treatment. Formation of antibodies to sheep erythrocytes was suppressed in CY-treated pigs until 10 days after immunization (6 days after the final CY treatment); control pigs showed a detectable response at 4 days after immunization. Six CY treatments prolonged the depression of all the above variables.

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