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IMMUNOSUPPRESSION WITH CYCLOPHOSPHAMIDE IN THE DOG

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SUMMARY

Cyclophosphamide significantly diminished the canine humoral antibody response to sheep red blood cells and tended to prevent arterial lesions in renal homografts. However, cyclophosphamide failed to prolong renal homograft survival when administered to dogs as the sole immunosuppressive agent, and it did not add to the effectiveness of azathioprine when given as a supplement to the azathioprine and administered simultaneously or sequentially.

INTRODUCTION

Although cyclophosphamide (Cytoxan^{R}) has been known for a number of years to be a superior immunosuppressive agent for the mouse (Berenbaum & Brown, 1964; Fox, 1964; Frisch & Davies, 1965), rat (Santos & Owens, 1962), guinea-pig (Calne & Leibowitz, 1963), rabbit (Brody, Jones & Haines, 1965; Jones *et al.*, 1963) and man (Santos *et al.*, 1970), it did not receive an extensive trial in a clinical transplantation program until relatively recently (Starzl *et al.*, 1971). The reluctance to treat human organ recipients with cyclophosphamide was at least partially attributable to its failure, as reported by Reams (1963) and Zukoski, Callaway & Rhea (1963), to prolong the survival of canine renal homografts, when used as the sole immunosuppressive agent.

The results in dogs with combination therapy have been less clear. In 1962, Calne, Alexandre & Murray had reported that the combination of cyclophosphamide and azathioprine, as opposed to azathioprine only, actually decreased the mean survival times of canine renal recipients as the result of increased bone marrow toxicity. However, MacPhee & Wright (1964) concluded that cyclophosphamide plus Actinomycin D prolonged the survival of canine lung homografts in comparison with untreated animals. Preston *et al.* (1966) came to much the same conclusion after treating recipients of intestinal homografts with cyclophosphamide and corticosteroids. Caves, Dong & Shumway (1973) in analysing their series of canine cardiac homotransplantations, found a significantly greater mean survival

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time when the recipients received cyclophosphamide in addition to horse antidog thymocyte globulin, than when they were treated with globulin only. However, using the same test systems, Kondo *et al.* (1974) were unable to demonstrate prolonged survival with the combination of cyclophosphamide and prednisone.

The purpose of the present study was to re-examine the potency of cyclophosphamide as an immunosuppressant in the dog by determining its ability, first, to inhibit the antibody response to sheep red blood cells, and second, to prolong renal homograft survival. In the latter trials, cyclophosphamide was administered both alone and with azathioprine.

MATERIALS AND METHODS

On day 0, 1 ml/kg body weight of washed sheep red cells (SRBC) resuspended in saline to 20% by volume was given intravenously to adult mongrel dogs. On the day before, and thereafter at about 2-day intervals, serum samples were obtained for the determination of anti-sheep erythrocyte antibody titres by a standard microplate method.

Group 1 (*six dogs*). Cyclophosphamide, 2 mg/kg body weight per day orally, was started 2 days before the primary immunizing dose of SRBC and continued for 13 days afterward.

Group 2 (*six dogs*). Two weeks after the first injection of SRBC a second dose was given intravenously. Cyclophosphamide was begun 2 days before the booster immunization. The cyclophosphamide dose was again 2 mg/kg orally, given daily for 2 weeks.

Group 3 (*six dogs*). Each dog received a primary and a secondary injection of SRBC spaced 2 weeks apart. No cyclophosphamide was given. These animals served as controls for groups 1 and 2.

Group 4 (*eight dogs*). Each dog received a primary injection of SRBC on the 3rd day after starting cyclophosphamide, 3 mg/kg/day orally.

Group 5 (*nine dogs*). Each dog received a primary injection of SRBC, but no cyclophosphamide. These animals served as controls for group 4.

TABLE 1. Mean survival times and incidence of leukopaenia in 128 canine recipients of renal homografts

		Treatment (mg/kg/day)							
		Days 0–4		Day 5 onward				Leukopaenia**	
Group	No. of dogs	Cyclo* (mg)	Aza† (mg)	Cyclo* (mg)	Aza† (mg)	Mean survival‡ ±s.d. (days)	*P* value§	No. of dogs	Percentage
A	11	3	—	3	—	12·5±5·2	NS	3	27
B	15	2	—	2	—	15·5±10·6	NS	3	20
C	11	2	1	2	1	17·0±7·7	NS	7	64
D	10	1	2	1	2	25·6±10·3	$P<0·005$	5	50
E	11	—	3	—	3	25·8±13·0	$P<0·01$	4	36
F	20	3	—	—	2	19·7±10·4	$P<0·05$	3	15
G	9	3	—	—	—	15·6±13·1	NS	0	0
H	10	—	—	—	2	17·6±13·2	NS	1	10
I	10	—	3	—	2	26·9±11·7	$P<0·005$	0	0
Controls	21	—	—	—	—	12·7±9·1		0	0

* Cyclo = cyclophosphamide (Cytoxan®).

† Aza = azathioprine (Imuran®).

‡ In computing the mean survivals, no dog was given credit for living more than 50 days.

§ Each group is compared to the control series.

** White blood cell count < 3000/mm³ on two consecutive determinations.

Renal homografts. Renal transplantation to the right iliac fossa with contemporaneous bilateral nephrectomy was performed in 159 dogs. In most instances, kidneys were exchanged between two recipients of different treatment groups. Alternatively, a common donor supplied kidneys to two recipients enlisted in different drug programmes. White blood cell counts and blood urea nitrogen levels were measured three times a week.

The treatment protocols for each of the ten groups are given in Table 1. Treatment was begun either on the day of operation (Groups A–G and I) or on the 5th post-operative day (Group H) and was continued until death in all groups but G, in which therapy was confined to a 5-day period.

Thirty-one animals dying within 5 days of transplantation were excluded from the study. For the purposes of statistical analysis, no dog was given credit for surviving more than 50 days; animals living beyond the arbitrary limit were killed.

At autopsy a sample of the homograft was taken, fixed in formalin and processed for light microscopy. Tissues from ninety-two of the 128 5-day survivals were available for histopathological analysis. The presence or absence of various features which are often associated with rejection were noted (Table 2).

TABLE 2. Analysis of histological lesions in ninety-two canine renal homografts treated with various combinations of cyclophosphamide and azathioprine

Type of lesion	Kidneys showing various histological changes in each treatment group* (%)								
	Control	A	C	D	E	F	G	H	I
Mononuclear cell infiltration of interstitium	100	71	40	40	42	80	86	90	40
Oedema of interstitium	100	57	30	29	67	67	100	90	40
Fibrinoid necrosis of walls of arterioles	86	14	14	14	18	50	75	50	10
Fibrinoid necrosis of walls of interlobular arteries	86	14	14	14	9	44	63	40	10

* See Table 1 for treatment schedules. Group B was not studied histologically.

Statistical analysis. In groups 1–3, haemagglutinin titres were measured on the same days in all dogs, so that a one-way analysis of variants with Student's *t*-test could be carried out. In addition, the growth curve technique of Grizzle & Allen (1969), in which comparisons are made after reducing the curves to polynomials, was used to compare the overall response curves for groups 1 and 2 to that of control group 3.

Since the haemagglutinin titres of animals in groups 4 and 5 were not always measured on the same days, the statistical methods described above could not be applied. Instead, polynomial regression curves were fitted to the haemagglutinin response curves of each dog and the resulting mean curves compared by the randomization test of G. Zerbe and S. Walker (unpublished data).

Statistical comparison of mean survivals of the homograft recipients in groups A to I and the control dogs were made with Student's *t*-test.

RESULTS

SRBC antibody response

The haemagglutinin response curves for the five groups of dogs are shown graphically in Figs 1 and 2. Those animals receiving 2 mg/kg of cyclophosphamide for 2 days before and for 13 days after the primary immunizing dose of red cells (group 1) appeared to show a partially suppressed antibody response when compared to the controls (group 3), but these differences were statistically significant ($P<0{\cdot}05$) only on days 5–11 (Fig. 1). When the over-

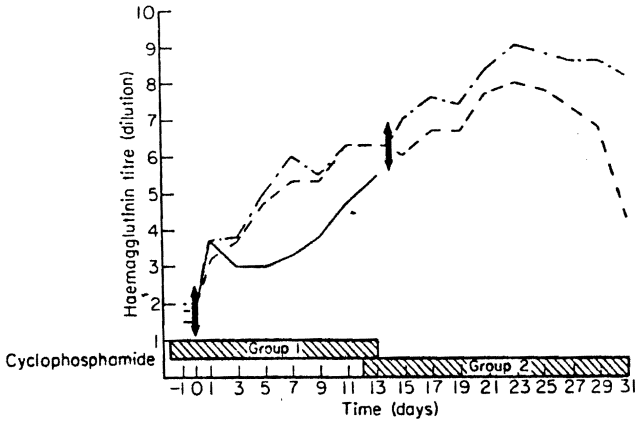

FIG. 1. Mean haemagglutinin titres for three groups of six dogs each after i.v. injections of SRBC (arrows). Cyclophosphamide (2 mg/kg orally) was given daily to dogs in groups 1 and 2 for the intervals indicated by the cross-hatched bars. (—) Group 1; (– – –) group 2; (— · — ·) group 3.

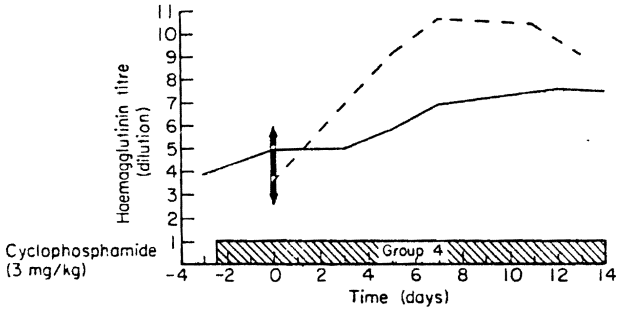

FIG. 2. Mean haemagglutinin titres after i.v. injections of SRBC (arrow). Group 4 (eight dogs) received cyclophosphamide (3 mg/kg orally) daily. The nine dogs of group 5 received no cyclophosphamide. (—) Group 4; (– – –) group 5.

all response curves of groups 1 and 3 were compared, they were not significantly different. Similarly, the anamnestic response to a second injection of SRBC (Fig. 1) seemed to be less forceful in the cyclophosphamide-treated dogs (group 2) than in the control animals (group 3). Although a day-by-day comparison revealed a significant difference only on day 31, the overall response curve of group 2 was significantly ($P<0{\cdot}01$) depressed in comparison to group 3. Dogs in group 4, which received 3 mg/kg/day of cyclophosphamide, similarly showed a diminution in the antibody response to SRBC cells (Fig. 2). This difference was significant at the $P<0{\cdot}01$ level when compared to the response curve of the control animals of group 5.

Renal homografts

Thirty-one recipients died within 5 days of operation and were eliminated from the study; the loss rate was approximately the same in each experimental group. The mean survival

times of the remaining 128 recipients are given for the nine treatment groups plus the controls in Table 1.

When cyclophosphamide was administered as the sole immunosuppressive agent in doses of 2 or 3 mg/kg (groups A, B and G), the post-operative survival was not significantly different from that of the controls.

In groups C and D, cyclophosphamide and azathioprine were given concomitantly, the total dosage being 3 mg/kg/day. The survival was significantly ($P<0{\cdot}005$) prolonged when the azathioprine component of therapy was 2 mg/kg, but not when the azathioprine dose was only 1 mg/kg.

Dogs in group F received cyclophosphamide for 5 days, then were switched to azathioprine. The mean survival time was prolonged (19·7 days, $P<0{\cdot}05$), but it was not significantly different from that of group H, which received only the azathioprine component of the treatment programme (17·6 days), and it was distinctly inferior to that of the recipients of groups E and I which were treated with azathioprine from the outset (25·8 and 26·9 days, respectively).

Leukopaenia (Table 1) was most frequent in animals receiving combination therapy (groups C and D) and in animals treated with 3 mg/kg of azathioprine continuously (group E). Nevertheless, a significant proportion of dogs receiving cyclophosphamide only for more than 5 days (groups A and B) were severely leukopaenic at the time of death.

Histopathological examination of the renal homografts (Table 2) showed that cyclophosphamide when administered as the sole immunosuppressive agent was ineffective if given at a dose of 3 mg/kg/day on days 0–4 only (group G). However, if treatment was continued for longer (group A) the incidence of fibrinoid necrosis of the walls of arterioles and small arteries in the grafts was greatly reduced although cellular infiltration was only slightly less.

When cyclophosphamide and azathioprine were given concomitantly (groups C and D) the frequency of both vascular lesions and cellular infiltration in the graft was greatly reduced. The combined effect of 2 mg/kg/day cyclophosphamide and 1 mg/kg/day azathioprine (group C) was as successful in preventing these lesions as a higher dose of azathioprine alone (groups E and I).

DISCUSSION

The present investigation confirms that cyclophosphamide is not protective of transplanted kidneys in the dog. In the thirty-five recipients of renal grafts treated with cyclophosphamide only, there was no prolongation of survival. In an additional forty-one dogs in which cyclophosphamide and azathioprine were combined in three different treatment schedules, the survival times appeared to mirror only the azathioprine component of therapy, with no demonstrable additive effect attributable to cyclophosphamide. However, histopathological analysis showed that although cyclophosphamide alone did not appreciably reduce the infiltration of the graft by mononuclear cells, a dose of 3 mg/kg/day did greatly decrease the incidence of fibrinoid necrosis of the walls of the arterioles and interlobular arteries. The selective inhibition of vascular lesions in the grafted kidneys is interesting, in view of the haemagglutinin antibody response data, because evidence is accumulating that these arteriolar and arterial changes are induced by humoral antibody (Jeannet *et al.*, 1970; O'Connell & Mowbray, 1973).

The failure to impair the cell-mediated component of the homograft reaction does not

fully explain the inability of cyclophosphamide to prolong graft survival in dogs. In the guinea-pig and mouse cyclophosphamide has been shown to suppress the function of B lymphocytes preferentially as compared with T lymphocytes (Poulter & Turk, 1972; Turk & Poulter, 1972). The same has been assumed to apply to humans. Yet in all three species, cyclophosphamide provides substantial protection to skin or renal homografts (Berenbaum & Brown, 1964; Calne & Leibowitz, 1963; Fox, 1964; Frisch & Davies, 1965; Maguire & Maibach, 1961; Starzl *et al.*, 1971).

One possible explanation for the inability of cyclophosphamide to protect homografts in the dog could be that the drug does not undergo efficient conversion in the canine liver, an obligatory step for its pharmacological activation. However, the enzymes required, the mixed function oxidase system of hepatic microsomes, are well represented in the dog, and it is known that the conversion of cyclophosphamide to its active form proceeds at least as rapidly in the dog as in man (Brock, 1967; Brock & Hohorst, 1963; Mellett, 1966).

Moreover, there is ample evidence that cyclophosphamide is not a pharmacological placebo in the dog, having both toxic (Lee, Castles & Kintner, 1973) and therapeutic effects. Leukopaenia (Schmidt *et al.*, 1964; Lee *et al.*, 1973; Moldovanu, Friedman & Miller, 1966; Pallotta, Rall & Ward, 1960; Storb et al., 1969), which was also documented in the present report, anaemia (Lee *et al.*, 1973; Pallotta *et al.*, 1960), haemorrhagic gastroenteritis (Epstein *et al.*, 1969; Lee *et al.*, 1973) and cystitis (Lee *et al.*, 1973; McClelland, 1963; Philips *et al.*, 1961), adrenal insufficiency (Tipton, 1964) and liver function abnormalities (Lee *et al.*, 1973) have all been ascribed in this species to cyclophosphamide administration. It has been used successfully in the dog as a diuretic (Zedeck & Mellett, 1964), in the treatment of malignant lymphomas (McClelland, 1963; Moldovanu *et al.*, 1966) and leukaemias (Moldovanu, 1964) and to ablate the bone marrow prior to haematopoietic engraftment (Epstein *et al.*, 1969; Storb *et al.*, 1969).

Despite its failure—for whatever reasons—as an immunosuppressant in the dog, cyclophosphamide has achieved, although perhaps belatedly, a recognized place in the pharmacological management of human organ recipients. This fact, in light of the negative results obtained in the dog, should caution against rigid adherence to a single test system and species in evaluating pharmacological agents of potential clinical applicability.

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