Clin. exp. Immunol. (1977) **30**, 309–316.

The effect of cyclophosphamide on an ocular immune response

I. PRIMARY RESPONSE

JOAN M. HALL, S. OHNO & J. F. PRIBNOW *The Francis I. Proctor Foundation for Research in Ophthalmology, University of California, San Francisco, California, and Naval Biosciences Laboratory, School of Public Health, University of California Berkeley, California, U.S.A.*

(*Received* 17 *May* 1977)

SUMMARY

The ocular inflammation and antibody production that follow intravitreal injection of rabbit eyes with bovine gamma globulin were suppressed by treatment with Cytoxan by the intramuscular (i.m.) route. The drug suppressed PFC responses of uveal tract and corneal cells when it was administered, beginning as late as 5 days after immunization, if treatment was continued until day 12 or 13. Short-term treatments and treatment with smaller Cytoxan doses were less effective. We noted a good correlation between the presence or absence of ocular inflammation, suppression of ocular PFC responses and depression of serum, aqueous humour and vitreous humour antibody titres. In some treatment groups ocular antibody production seemed to be completely suppressed, while in others antibody production was significantly delayed.

INTRODUCTION

Cyclophosphamide (Cytoxan), a synthetic antineoplastic drug usually classified as an alkylating agent, has been used experimentally as an immunosuppressive drug in mice (Bohunická *et al.*, 1972; Poulter & Turk, 1972; Stockman *et al.*, 1973; Lagrange, Mackaness & Miller, 1974; Askanese, Hayden & Gershon, 1975; Jokipii & Jokıpii, 1973; Kawaguchi, 1970; Stockman & Trentin, 1972; Hoffsten & Dixon, 1974), guinea-pigs (Revell, 1974; Polak & Turk, 1974) and rats (Santos, 1967). Single doses of Cytoxan (250–400 mg/kg) and smaller divided doses, given either intraperitoneally or intravenously, suppressed normal anti-sheep erythrocyte responses in mice. Unlike some other immunosuppressive agents, cyclophosphamide was effective even when given after antigen (Kawaguchi, 1970; Santos, 1967).

The effect of cyclophosphamide on the response to antigens other than erythrocytes has been studied in mice. Kawaguchi (1970) suppressed the response to bovine gamma globulin, and Stockman & Trentin (1972) studied the effect of the drug on the response to equine gamma globulin. Hoffsten & Dixon (1974) investigated its suppressive effects on anti-keyhole limpet haemocyanin production.

The immunosuppressive effects of Cytoxan have not been investigated extensively in rabbits. Nakamura & Weigle (1970) suppressed thyroid lesions in rabbits by giving Cytoxan after the induction of thyroiditis. Others have studied the effect of the drug on skin or renal grafts (Jasani, 1973; Jones *et al.*, 1963; Friedman *et al.*, 1973), or on rabbit bone marrow cells (Kissling & Speck, 1973).

Our previous studies (Hall & Pribnow, 1972) indicated that the cells of the rabbit uveal tract produce antibody after the intravitreal injection of protein antigens. The greatest number of uveal tract plaque-forming cells (PFC) were found between 14 and 20 days after immunization. The purpose of the present work was to determine whether Cytoxan would suppress the antibody production and ocular inflammation that follow intravitreal injection of bovine gamma globulin. These experiments took advantage of the fact that Cytoxan can be given intramuscularly (i.m.) without causing local tissue necrosis.

Correspondence: Dr Joan M. Hall, Francis I. Proctor Foundation, University of California, San Francisco, California 94143, U.S.A.

MATERIALS AND METHODS

Experimental animals and immunizations. We used New Zealand white male rabbits weighing 3·0–3·5 kg each. We injected the right eye of each rabbit with 1·5 mg bovine gamma globulin (BGG, Miles Laboratories, Kankakee, Illinois). The BGG, dissolved in pyrogen-free saline (McGaw & Co., Glendale, California) and sterilized by filtration (0·45 μm filter), was prepared just prior to its injection.

The rabbits were bled from the marginal ear vein before immunization and on days 7, 10, 12, and 14. Those killed on day 21 were bled on days 16, 19 and 21 also. The heat-inactivated serum was adsorbed with sheep erythrocytes and frozen until used in the antibody titrations.

Cytoxan treatment. We injected Cytoxan (Mead–Johnson, Evansville, Indiana) i.m. every other day beginning at various times relative to the BGG injections. Except in the pre-treated rabbits, the dose was 100 mg per injection. The rabbits were given the antibiotic Tylocine (Eli Lilly & Co., Indianapolis, Indiana) to prevent the overgrowth of opportunistic bacterial pathogens.

Pre-treatment groups. We injected 200 mg Cytoxan 2 days before the intravitreal injection of BGG and immediately before immunization. The dose on the subsequent days was 100 mg. The rabbits were killed 14 days after immunization. A group of five rabbits received 100 mg Cytoxan just before the BGG injections and every other day until day 12.

Post-treatment groups. We treated these rabbits beginning 2, 3, 4 or 5 days after immunization and continued the treatment until day 12 or 13. Some rabbits in each group were killed 14 days after immunization and some on day 21.

Short-term treatment groups. We treated one group of rabbits on days 2, 4 and 6, and a second group on days 2, 4, 6 and 8. The animals were killed either 14 or 21 days after the BGG injections.

Low dose groups. Seven rabbits received 50 mg Cytoxan every other day beginning on day 2 and continuing until day 12. The total dose was 300 mg. Three rabbits were killed on day 14, and four on day 21.

Control rabbits. At least one control rabbit was immunized at the same time as each group of treated rabbits. The controls received i.m. injections of saline and Tylocine. The results of the plaque assays on the tissues of all control rabbits were similar and have been pooled.

Plaque assays and antibody determinations. We determined the number of PFC in the lymph nodes and uveal tracts of all rabbits by the modified plaque assay described previously (Hall & O'Connor, 1970). The numbers of PFC in the corneal (limbal) tissue of some rabbits was also determined. Plaque numbers are expressed as PFC per 10^6 cells in a given suspension.

Antibody titres in the serum (from each bleeding), and in the aqueous and vitreous humour from each injected eye, were assayed by the plate haemolysin test (Hall, 1971). Titres are expressed as the $\log_2$ of the reciprocal of the highest dilution of a sample that showed haemolysis.

The effect of Cytoxan on peripheral leucocytes. Two rabbits injected with BGG received 100 mg Cytoxan on days 0, 2 and 4. They were bled every day and the total leucocyte count determined. We also performed differential counts on Giemsa-stained smears. Two other intravitreally immunized rabbits received the Cytoxan on days 2, 4, 7, 9 and 11. Leucocyte counts and differential counts were done on the days the Cytoxan was injected.

Statistical analysis. We used the two-sample rank test (Goldstein, 1964) to compare the PFC responses in treated and control rabbits. This non-parametric test was used because it requires no assumptions as to the normality of the distribution of the values. A P value of 0·05 was considered significant.

RESULTS

Toxicity of Cytoxan

The Cytoxan produced only minor toxicity when it was injected i.m. A few rabbits developed transient diarrhoea with accompanying weight loss. Premature deaths from diarrhoea or other causes were generally prevented if the rabbits were not used until they had been in the animal care facility for at least 1 week after their arrival. In subsequent experiments we found that the Tylocine dose could be decreased, and the drug given less frequently, with no increase in the incidence of toxic reactions.

Effect of Cytoxan on peripheral leucocytes

In all rabbits the number of polymorphonuclear neutrophiles (PMN) rose initially and then declined and remained low for several days. The numbers of lymphocytes were not so profoundly affected. Three of the rabbits showed a suppression of the immune response to BGG. Figs 1 and 2 show the numbers of total leucocytes, lymphocytes, and PMN in two of the treated rabbits.

Effect of Cytoxan on ocular inflammation, plaque assay and antibody titres

Rabbits killed on day 14. Control rabbits developed uveitis on day 7. The inflammation persisted for several days. The eyes of the rabbits that received Cytoxan up to day 12 or 13 remained essentially normal

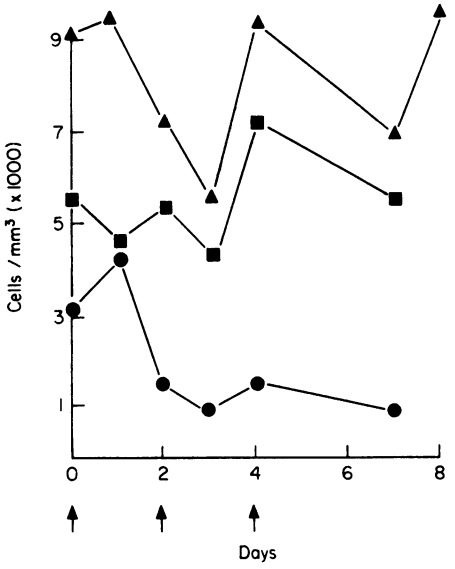

FIG. 1.

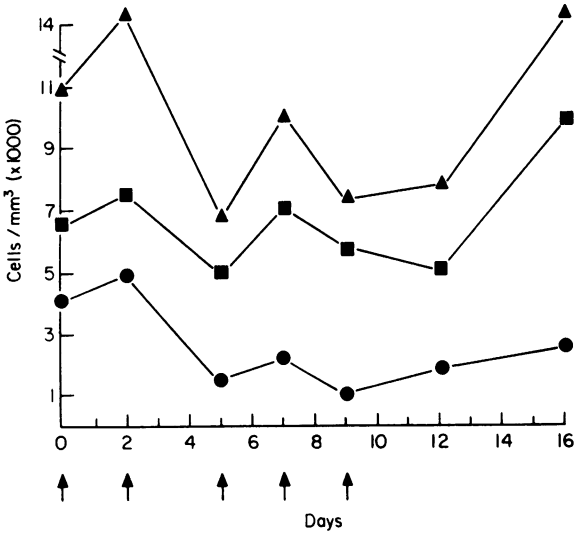

FIG. 2.

FIG. 1. Leucocyte counts of a rabbit that received 300 mg Cytoxan intramuscularly. Arrows indicate days of Cytoxan injections, BGG was injected intravitreally on day 0. (▲) Total leucocytes; (■) lymphocytes; (●) PMN.

FIG. 2. Leucocyte counts of a rabbit that received 500 mg Cytoxan intramuscularly. Arrows indicate days of Cytoxan injections. BGG was injected intravitreally on day 0. (▲) Total leucocytes; (■) lymphocytes; (●) PMN.

throughout the observation period. The results of the plaque assays on the tissues of the rabbits killed on day 14 are presented in Table 1. Cytoxan significantly suppressed the PFC response in the uveal tracts and corneas when the first administration of Cytoxan occurred before immunization or as late as 5 days afterward. Only five out of thirty-eight treated rabbits had ocular PFC, and in four of these the numbers were low. Eight of the control rabbits had more than 1000 PFC per 10^6 uveal tract cells. One rabbit in the 5–13-day treatment group had more than 4000 uveal tract PFC per 10^6 cells.

The difference between the PFC numbers in the lymph nodes of treated and control rabbits was statistically significant in the groups in which treatment began on day 0, 2, 3 or 5. In most groups about half of the rabbits had lymph node PFC.

Cytoxan was not so effective when it was given for a short time early in the immune response. Some rabbits in each group developed uveitis by 7 days after immunization, and had PFC in their uveal tracts and corneas. The uveal tract responses of the rabbits that received Cytoxan on days 2 through to 8 were significantly suppressed, but the responses of the rabbits that were treated on days 2, 4 and 6 were not. Neither group showed significant suppression of the PFC response in the lymph nodes.

The three rabbits that received 50 mg doses of Cytoxan all developed uveitis, and the PFC responses in the uveal tracts and corneas were not significantly suppressed (Table 2). All three had antibody in the aqueous humour and in the serum (Table 3).

As seen in Table 4, there was no detectable antibody in the serum, in the aqueous humour or in the vitreous humour of most rabbits that received Cytoxan through to day 12 or 13. These rabbits had had no ocular PFC. The one exception was the rabbit in the 5–13-day treatment group that showed ocular tissue PFC. Serum antibody was detected in most of the control rabbits by day 7, and in all controls by day 10. Some rabbits in each short treatment group had serum and aqueous humour antibody titres.

Rabbits killed on day 21. The eyes of the rabbits whose treatment began on day 2 remained normal throughout the experiment. The uveal tract and corneal PFC responses of the rabbits were significantly suppressed (Table 5). We also obtained significant suppression of the ocular response when we began treatment on day 3 or 4. Some rabbits in each of the two groups developed uveitis several days after the

TABLE 1. PFC per 10^6 cells in tissues of Cytoxan-treated rabbits killed 14 days after intravitreal injection of BGG

Treatment schedule (days)	Total dose (mg)	PFC per 10^6 cells		
		Uvea	Cornea	Lymph node
None	0	1971* (12/12)† (529–6000)‡	1299 (6/6) (100–3166)	66 (11/11) (14–181)
−2–12	1000	0 (0/8)	n.d.	66 (3/6)
0–12	700	0 (0/5)	0 (0/2)	8 (1/5)
2–12	600	0 (0/7)	0 (0/4)	25 (4/7)
3–13	600	3 (1/6) [17]§	0 (0/6)	16 (3/6)
4–12	500	22 (2/6) [7,125]	0 (0/6)	24 (3/6)
5–13	500	742 (2/6) [11,4444]	65 (1/6) [388]	6 (4/6)
2–6	300	921 (2/4) [685,3000]	673 (2/4) [1214,1470]	64 (4/4)
2–8	400	8 (2/5) [14,27]	2 (1/5) [12]	159 (4/5)

n.d. = Not done. Discrepancies in total numbers of tissues assayed in cornea and uveal tracts of some groups are due to the fact that in the first experiment corneas were not assayed.

* Numbers represent arithmetic means for all tissues from a particular treatment group.

† Numbers of tissues with PFC per total number of tissues assayed.

‡ Range for tissues of control rabbits.

§ Numbers in brackets are plaque numbers of the individual rabbits that responded. All other tissues in group had no PFC.

TABLE 2. PFC per 10^6 cells of rabbits that received six × 50 mg doses of Cytoxan

Day killed	Group	PFC per 10^6 cells		
		Uvea	Cornea	Lymph node
14	Control	1971 (12/12)*	1299 (6/6)	66 (11/11)
	Treated	2522 (3/3)	2249 (3/3)	40 (3/3)
21	Control	1099 (6/6)	1088 (6/6)	31 (6/6)
	Treated	423 (2/4) [3160, 192]†	838 (2/4) [278, 1416]	10 (4/4)

* Numbers of tissues with PFC per total number of tissues assayed.

† Plaque numbers of individual rabbits that responded. Other tissues in group had no PFC.

TABLE 3. Haemolytic antibody titres in serum, aqueous humour and vitreous humour of rabbits that received 50 mg doses of Cytoxan

Day killed	Group	Antibody titre (log_2)		
		Serum	Aqueous	Vitreous
14	Control	4·9	8·0	0·5
	Treated	5·7	7·6	0
21	Control	3·2	8·8	12·5
	Treated	0·5	0	3·7

* Numbers represent arithmetic means.

TABLE 4. Haemolytic antibody titres in serum, aqueous humour and vitreous humour of rabbits killed 14 days after intravitreal injection of BGG

Treatment Group	Antibody titre (log_2)		
	Serum	Aqueous	Vitreous
Control	4·9* (12/12)†	8 (12/12)	0·3 (1/12)
−2–12	0‡	0	0
0–12	0	0	0
2–12	0	0	0
3–13	0	0	0
4–12	0	0	0
5–13	0·5 (1/6)	2·6 (1/6)	0
2–6	2·0 (2/4)	5·5 (2/4)	0
2–8	2·0 (2/5)	0	0

* Numbers represent arithmetic means.

† Numbers in parentheses refer to number of samples with antibody per total number of samples.

‡ '0' Indicates no detectable antibody in undiluted samples.

last Cytoxan injection, and had ocular PFC. The sample numbers in the 5–13-day group and the short treatment groups were not large enough to permit accurate statistical analysis. The rabbits that had ocular PFC (Table 5) also developed uveitis. There was no significant suppression of the lymph node PFC responses of any of the rabbits.

Half of the rabbits that received 50 mg doses of Cytoxan showed PFC in the ocular tissues (Table 2). The suppression was not statistically significant. Serum, aqueous and vitreous antibody titres were reduced (Table 3).

As shown in Table 6, the antibody titres in the serum and ocular fluids of the Cytoxan-treated rabbits killed on day 21 were reduced. There was no antibody in rabbits whose treatment began on day 2. One animal in each of the other groups had aqueous antibody. Vitreous antibody in most rabbits was completely absent.

DISCUSSION

These experiments showed that i.m. injections of 100 mg of cyclophosphamide given every other day suppressed the primary immune response of rabbits to intravitreally injected BGG. The ocular PFC response was either completely suppressed or significantly reduced, the antibody titres in the serum and

TABLE 5. PFC per 10^6 cells in tissues of Cytoxan-treated rabbits killed 21 days after intravitreal injection of BGG

Treatment schedule	Total dose (mg)	PFC per 10^6 cells		
		Uvea	Cornea	Lymph node
None	0	1009* (6/6)† (208–2772)‡	1088 (6/6) (215–3583)	31 (6/6) (1–54)
2–12	600	0 (0/3)	0 (0/3)	8 (3/3)
3–13	600	23 (1/4) [92]§	2 (1–4) [9]	18 (2/4)
4–12	500	132 (3/4) [53, 66, 409]	175 (3/4) [24, 119, 555]	48 (3/4)
5–13	500	163 (1/3) [490]	63 (1/3) [188]	35 (3/3)
2–6	300	167 (2/2) [31, 312]	100 (2/2) [201]	14 (1/2)
2–8	400	114 (2/2) [40, 188]	60 (1/2) [121]	49 (2/2)

* Numbers represent arithmetic means for all tissues from a particular treatment group.

† Numbers of tissues with PFC per total number of tissues assayed.

‡ Range for tissues of control rabbits.

§ Numbers in brackets are plaque numbers of the individual rabbits that responded. All other tissues in group had no PFC.

TABLE 6. Haemolytic antibody titres in serum, aqueous humour and vitreous humour of rabbits killed 21 days after intravitreal injection of BGG

Treatment group	Antibody titre ($\log_2$)		
	Serum	Aqueous	Vitreous
Control	3·2* (6/6)†	8·8 (6/6)	12·5 (6/6)
2–12	0‡	0	0
3–13	0	2·0 (1/4)	0
4–12	0·8	4·0 (1/4)	0
5–13	0	1·0 (1/3)	0
2–6	2·0 (2/2)	1·5 (2/2)	0
2–8	2·5 (2/2)	3·0 (1/2)	4·0 (1/2)

* Numbers are arithmetic means.

† Numbers in parentheses refer to number of samples with antibody per total number of samples.

‡ '0' Indicates no detectable antibody in undiluted samples.

in the aqueous and vitreous humours were depressed, and ocular inflammation was absent or delayed in onset. Cytoxan was effective even when treatment was begun several days after immunization if it was continued until day 12 or 13.

The immunosuppressive effects of Cytoxan in mice and guinea-pigs are well-known. Bohunická *et al.* (1972) and Kawaguchi (1970) noted that Cytoxan suppressed primary responses even when it was given after antigen. Santos (1967) suppressed humoral immunity in rats by treating them with Cytoxan after immunization. Kawaguchi (1970) believed that antigen-sensitive cells, when stimulated to multiply,

may be especially sensitive to Cytoxan. Hoffsten & Dixon (1974) were able to depress an established immune response to keyhole limpet haemocyanin.

The mechanism of immune suppression by Cytoxan has been studied in mice and guinea-pigs. Turk & Poulter (1972), Turk, Parker & Poulter (1972) and Jokipii & Jokipii (1973) found that lymphocytes in the non-thymus-dependent areas of the lymph nodes and spleens of mice and guinea-pigs were depleted. Poulter & Turk (1972) and Revell (1974) noted a proportional increase in the theta-carrying lymphocytes of peripheral lymphoid tissue after treatment with cyclophosphamide. Turk, Parker & Poulter (1972) stated that Cytoxan was selectively cytotoxic to short-lived cells of peripheral lymphoid tissue. In addition to the evidence that Cytoxan affects B lymphocytes, there is also evidence that it affects T-cell function. Enhancement of delayed hypersensitivity reactions is thought to be due to the action of Cytoxan on suppressor T cells (Askanese *et al.*, 1975; Mitsuoka, Baba & Morikawa, 1976). Milton *et al.* (1976) have described the effects of cyclophosphamide on several T-cell functions.

Our results were similar to those found in mice and guinea-pigs. The dose of Cytoxan we used (approximately 30 mg/kg) was chosen after preliminary experiments showed that 50 mg given every 5 days did not alter the immune response to intravitreally injected BGG. The 100 mg dose significantly suppressed the PFC response in ocular tissues, and in some cases also suppressed the lymph node response. Smaller doses given at the same intervals were less effective. In general, we noted good correlation between the presence or absence of uveitis and the results of the plaque and antibody assays.

Our previous experiments (Hall & Pribnow, 1972) indicated that antigen can be detected in the vitreous humour for at least 14 days after injection. The results of the short-term experiments would seem to indicate that Cytoxan is more effective if it is given during the time that the antigen is in the vitreous humour and available to the immunocompetent cells that migrate to the eye. Some rabbits that received the short-term treatment (days 2–8) did, however, show significantly reduced numbers of ocular PFC on day 14, and none had aqueous humour antibody. The fact that the total dose was smaller in these groups must also be considered.

Rabbits were killed approximately 1 week after Cytoxan treatment was stopped (day 21), to determine whether the drug actually suppressed the immune response or merely delayed its onset. The rabbits treated on day 2 through to 12 did not develop ocular inflammation, had no PFC in ocular tissues and no antibody in serum or ocular fluids. PFC were found in the ocular tissues of some rabbits in the other groups (treatment beginning on day 3, 4 or 5), indicating that antibody formation could have been delayed in these rabbits. The rabbits that were able to mount an immune response developed uveitis 3 or 4 days after the Cytoxan treatment ended. Most of them had antibody in the aqueous humour, but none had vitreous humour antibody, another indication that antibody formation was delayed. Vitreous antibody usually appears about 1 week after the onset of inflammation. The rabbits in the short treatment groups all developed uveitis and had ocular PFC. The antibody titres in serum and aqueous humour were depressed.

We might hyopthesize that in our experimental system, Cytoxan is suppressing the immune response by acting directly on B cells that have been stimulated to multiply by the BGG. Although we have no direct evidence, we cannot rule out the possibility that a necessary T-cell function is also altered by the Cytoxan. The fact that the drug is more effective when given for several days during the course of the response might indicate that short-lived, rapidly dividing cells are the elements most affected.

In most groups we noted some lymph node PFC, even though there were no ocular PFC and no serum, aqueous or vitreous antibody in the same rabbits. These results further substantiate our claim that the majority of the antibody present after intravitreal injection of protein antigens is produced locally by the ocular cells.

The fact that Cytoxan was effective even when treatment was begun several days after immunization could have important clinical applications in the treatment of some forms of uveitis. Patients would clearly present themselves to the ophthalmologist after encountering an antigenic stimulus.

This work was supported in part by grants No. EY-10182 and EY-1597 from the national Eye Institute, National Institutes of Health; and by the Office of Naval Research under a contract between the Office of Naval Research and the Regents of the University of California.

REFERENCES

ASKANESE, P.W., HAYDEN, B.J. & GERSHON, R.K. (1975) Augmentation of delayed type hypersensitivity by doses of cylophosphamide which do not affect antibody responses. *J. exp. Med.* **141**, 697.

BOHUNICKÁ, E., KOZA, F., KŮZELA, Š., KREMPASKÝ, U. & UJHÁZY, V. (1972) Immunosuppression with cyclophosphamide: effect on antibody plaque forming spleen cells and on hemolytic antibodies production. *Neoplasma*, **19**, 611.

FRIEDMAN, E.H., UENO, A., BEYER, M.M. & NICASTRI, A.D. (1973) Combination drug treatment in immunosuppression. Effect of azathioprine, cyclophosphamide and methylprednisolone on rabbit renal allografts. *Transplantation*, **15**, 619.

GOLDSTEIN, A. (1964) *Biostatistics, an Introductory Text.* MacMillan Company, New York.

HALL, J.M. (1971) Specificity of antibody formation after intravitreal immunization with bovine gamma globulin and ovalbumin. I. Primary response. *Invest. Opthal.* **10**, 775.

HALL, J.M. & O'CONNOR, G.R. (1970) Correlation between ocular inflammation and antibody production. II. Hemolytic plaque formation by cells of the uveal tract. *J. Immunol.* **104**, 440.

HALL, J.M. & PRIBNOW, J.F. (1972) Responses of the reticuloendothelial system of the rabbit to intraocular injections of foreign protein. *Cell. Immunol.* **5**, 419.

HOFFSTEN, P.F. & DIXON, F.J. (1974) Effect of irradiation and cyclophosphamide on anti-KLH antibody formation in adult mice. *J. Immunol.* **112**, 564.

JASANI, K.K. (1973) A new approach for studying the influence of cyclophosphamide upon the rejection of rabbit skin homografts. *Brit. J. Pharmac. Chemother.* **48**, 334.

JOKIPII, A.M.M. & JOKIPII, L. (1973) Suppression of cell-mediated immunity by cyclophosphamide: its independence of concomitant B cell response. *Cell. Immunol.* **9**, 477.

JONES, J.W., BRADY, G.L., ONEAL, R.M. & HAINES, R.F. (1963) Prolongation of skin homografts in rabbits using cyclophosphamide. *J. Surg. Res.* **3**, 189.

KAWAGUCHI, S. (1970) Studies on the induction of immunological paralysis to bovine gamma globulin in adult mice. II. The effect of cyclophosphamide. *Immunology*, **19**, 26.

KISSLING, M. & SPECK, B. (1973) The effect of cyclophosphamide, ^{32}P, and ^{60}Co irradiation on rabbit bone marrow cells. *Blut*, **27**, 167.

LAGRANGE, P.H., MACKANESS, G.B. & MILLER, T.E. (1974) Potentiation of T cell mediated immunity by selective suppression of antibody formation with cyclophosphamide. *J. exp. Med.* **139**, 1529.

MILTON, J.D., CARPENTER, C.B. & ADDISON, I.E. (1976) Depressed T-cell reactivity and suppressor activity of lymphoid cells from cyclophosphamide treated mice. *Cell. Immunol.* **24**, 308.

MITSUOKA, A., BABA, M. & MORIKAWA, S. (1976) Enhancement of delayed hypersensitivity by depletion of suppressor T cells with cyclophosphamide in mice. *Nature (Lond.)*, **262**, 77.

NAKAMURA, R.M. & WEIGLE, W.O. (1970) Suppression of thyroid lesions in rabbits by treatment with cyclophosphamide after the induction of thyroiditis. *Clin. exp. Immunol.* **7**, 541.

POLAK, L. & TURK, J.L. (1974) Reversal of immunological tolerance by cyclophosphamide through inhibition of suppressor cell activity. *Nature (Lond.)*, **249**, 654.

POULTER, L.W. & TURK, J.L. (1972) Proportional increase in the O carrying lymphocytes in peripheral lymphoid tissue following treatment with cyclophosphamide. *Nature, New Biol.* **238**, 17.

REVELL, P.A. (1974) Studies on the effect of cyclophosphamide on T and B lymphocytes in the blood, lymph nodes and thymus of normal guinea-pigs. *Int. Arch. Allergy appl. Immunol.* **47**, 864.

SANTOS, G.W. (1967) Immunosuppressive Drugs. I. *Fed. Proc.* **26**, 907.

STOCKMAN, G.D., HEIM, L.R., SOUTH, M.A. & TRENTIN, J.J. (1973) Differential effects of cyclophosphamide on the B and T cell compartments of adult mice. *J. Immunol.* **110**, 277.

STOCKMAN, G.D. & TRENTIN, J.J. (1972) Cyclophosphamide-induced tolerance to equine globulin and to equine anti-mouse thymocyte globulin in adult mice. I. Studies on antigen and drug requirement. *J. Immunol.* **108**, 112.

TURK, J.L. & POULTER, L.W. (1972) Selective depletion of lymphoid tissue by cyclophosphamide. *Clin. exp. Immunol.* **10**, 285.

TURK, J.L., PARKER, D. & POULTER, L.W. (1972) Functional aspects of the selective depletion of lymphoid tissue by cyclophosphamide. *Immunology*, **23**, 493.