Suppression of the Graft-Versus-Host (GVH) Reaction in Chick Embryos by Cyclophosphamide[1]

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INTRODUCTION

Grafts from allogeneic donors are regularly rejected by immunologically competent individuals despite the presence of lymphoid cells capable of reacting against host tissues. When recipient animals are unable to defend themselves against the immunologically competent cells of foreign grafts, a graft-versus-host (GVH) reaction ensues which is manifested by spleno-hepatomegaly, runting and death of the hosts. This occurs when the host is (1) immunologically immature, (2) neonatally thymectomized, (3) sublethally or lethally irradiated, (4) treated with immunosuppressant drugs, or (5) F_1 grafted with parental cells (Simonsen, 1957; Schwartz et al., 1957; Billingham, 1959; Aisenberg, et al., 1962; Owens and Santos, 1968).

Many anticancer drugs can suppress antibody formation and prolong survival of transplanted organs. Their application may modify the severity of the GVH reaction that often accompanies the grafting of lymphoid tissues (Barnes et al., 1966) and like X-irradiation may result in prolonged chimerism (Glynn et al., 1968). The present experiments were conducted to assess the effectiveness of cyclophosphamide, a cytostatic drug, in reducing the severity of the GVH reactions in chick embryo hosts and in inhibiting the development of transplantation immunity in baby chicks.

MATERIALS AND METHODS

Juvenile and adult White Leghorn and adult Game chickens served as blood donors and White Leghorn embryos as recipients. All fertile eggs and chickens were obtained from a local source.

Embryonic splenomegaly assay—Of several criteria commonly used to measure the GVH reaction—host mortality, runting, hepatomegaly, and splenomegaly—the last was the most consistent and quantitatively measurable index (Seto, 1968a). Of several tissues tested, blood was convenient to handle and most consistent in eliciting the GVH reaction. Donor blood, obtained by cardiac puncture in equal volume of 1% sodium citrate solution or Alsever's solution, was centrifuged and enough supernatant citrate-plasma was discarded to restore the original blood concentration. After thoroughly mixing, 0.1 ml of blood was injected intravenously into each 13- to 14-day White Leghorn recipient. The embryos were killed five days later for body, liver and spleen weight determinations. The body weight, a measure of runting, the liver to body-weight ratio (liver index), a measure of hepatomegaly, and spleen weight, a measure of splenomegaly, provided quantitative parameters of the GVH reaction.

Effects of cyclophosphamide (CX) *on the GVH reaction*—Blood from adult Game chicken donors was exposed *in vitro* for 5 to 10 min to differ-

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ent concentrations (0.13 to 2.0 mg/ml of blood) of the drug. The blood cells were centrifuged, washed to remove the agent, and resuspended in equivalent volume of sterile Tyrode or citrate solution. Untreated blood, processed in a similar manner, served as the control. The GVH competence of treated and untreated blood was assessed with the embryonic splenomegaly assay.

In a second experiment, 4 of 5 groups of recipient embryos were treated with 1 mg of CX/10 g embryo weight, at 12 hr, 1, 2, and 3 days after the inoculation of Game chicken blood, and the remaining untreated group served as the positive control. The splenomegaly produced in the different groups was compared.

In a third experiment, embryos were injected with single injections of graded doses of CX, ranging from 0.5 to 4.0 mg/embryo, a day before the grafting of donor cells. Other groups of embryos received either blood or CX only. Six days later the embryos were killed for body and organ weight determinations.

Effect of CX treatment on the cellular immune potential of donors — White Leghorn embryos, 17 to 18 days old, were inoculated intravenously with 1-2 mg of CX in 0.1 ml of Tyrode's solution and after hatching blood samples were collected by cardiac puncture at intervals for 11 weeks and assayed for the GVH reaction capacity. Blood from untreated White Leghorn donors of similar ages was also tested.

RESULTS

Effects of cyclophosphamide on the GVH reaction — The effects of increasing CX concentrations upon the capacity of donor cells to elicit the GVH reaction are summarized in Figure 1 (A,C,E). The splenomegaly was greatest among hosts inoculated with blood exposed to the lowest concentration of drug tested.. This apparent stimulatory effect upon exposure to low concentrations of toxic agents has been observed frequently in other biological systems. The group eliciting the greatest GVH reaction was designated as the 100% level and the others were compared to it. The decrease in competence of donor calls, as measured by the three parameters of the GVH reaction, was generally proportional to exposure to increasing concentrations of the agent.

The effect on the GVH reaction of exposure of host embryos to increasing concentrations of CX is summarized in the other half of Figure 1 (B,D,F). Since CX treatment itself reduces the body and organ weights, the mean spleen weights of CX-treated host embryos were compared with those of CX-treated embryos rather than with sham-treated embryos. The mean splenomegaly of non-CX-treated grafted hosts was arbitrarily designated as the 100% level and that of CX-treated hosts was measured relative to it. Splenomegaly and hepatomegaly decreased with increasing drug doses. The apparent increase in runting at the higher CX doses is probably attributable more to the growth-suppressive effect of the drug than to an elevated GVH reaction.

The effects of delaying the CX treatment of hosts for varying periods after inoculation of donor blood are shown in Table I. Compared with hosts inoculated only with blood, recipients exposed to CX 12 hr to 3 days after grafting show significant reduction of splenomegaly. Both donor and host components involved in the organ enlargement are susceptible to the drug in this situation.

Effect of cyclophosphamide on the cellular immune potential of donors — The growth of the GVH capacity during the first 11 weeks is shown in Figure 2 for untreated and CX-treated White Leghorn donors. The cellular immune potential of donor blood, as measured by host-embryo splenomegaly, is plotted as the function of donor age. The numbers of

donors are shown for both groups but the frequency of responding embryo hosts is given only for the drug-treated group since related data for untreated donors were reported elsewhere (Seto, 1968b). The GVH competence of the experimental group is significantly lower than that of control donors in the first few weeks. The near normal level of competence attained by the 9th week indicates that considerable recovery from the immunosuppression has occurred.

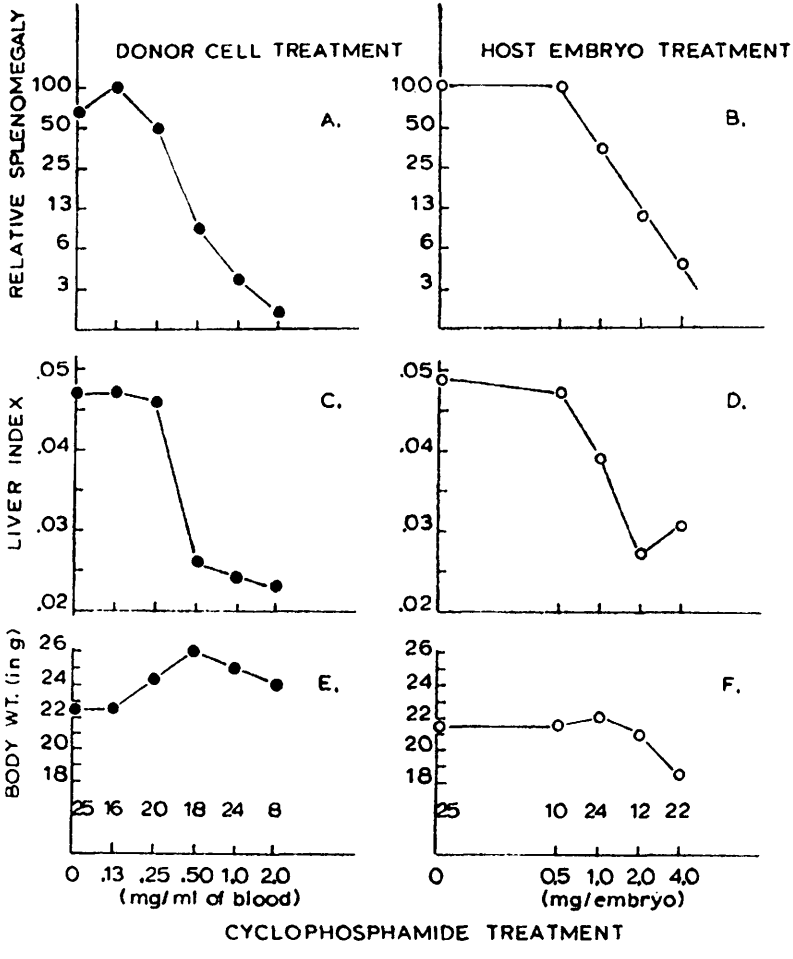

Figure 1. Effect of cyclophosphamide treatment of donor blood cells (●) and of host embryos (o) on the graft-versus-host (GVH) reaction. Three parameters of the GVH reaction (relative splenomegaly, liver index and body weight) are plotted as a function of CX doses. Sample sizes are given above the CX doses in curves E and F.

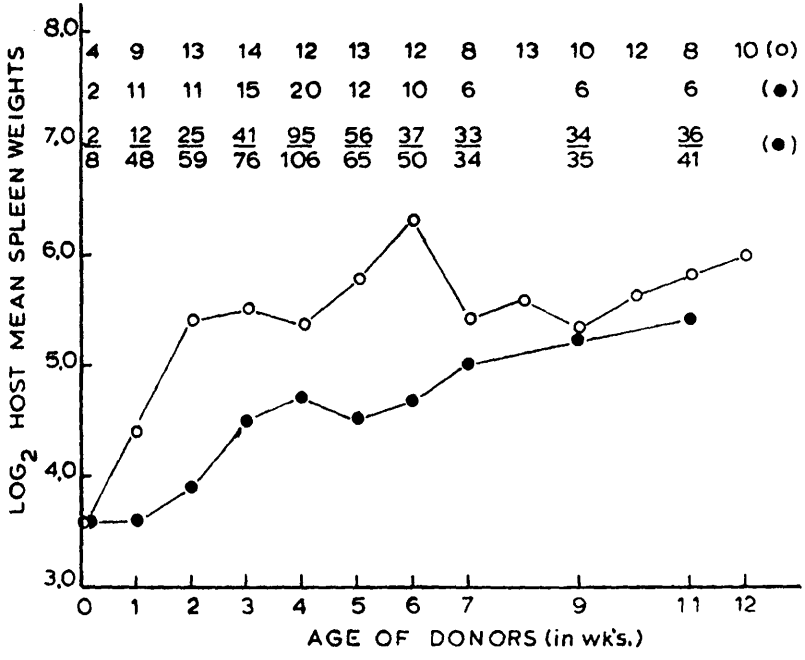

Figure 2. Comparison of the growth of the GVH reaction capacity of untreated White Leghorn donors (o) and of donors exposed to CX as embryos (●).

Discussion

A number of antineoplastic drugs (Karnofsky, 1968) have been used as immunosuppressants with varying degrees of success (Levy, 1966; Santos, 1967; Wheeler, 1967). Among the most effective are the alkylating agents of which cyclophosphamide is a clinically important example (Fox, 1964; Santos, 1967). When administered in sufficient amounts, it can suppress both tissue and humoral immunological responses (Owens and Santos 1968) and in animals grafted with hemopoietic tissues may result in GVH reactions and extended chimerism (Glynn et al., 1968).

The findings reported here indicate that cyclophosphamide is very effective in suppressing the GVH reaction in grafted chick embryos and in delaying the development of tissue immunity in chickens. Exposure of donor cells *in vitro* to cyclophosphamide reduces the capacity for GVH reaction. The dose-dependent relationship is similar to that reported for *in vitro* X-irradiation of donor cells in GVH tests (Kryukova, 1959; Seto and Albright, 1965). The reduced competence of donor cells exposed to X-irradiation or to CX is probably related to the decrease in proliferative capacity of the cells. The exposure of embryo hosts to graded doses of CX before the inoculation of donor cells resulted in a similar dose-dependent decrease in the GVH reaction. This dose-response relationship has been observed also when host embryos were X-irradiated (Seto and Albright, 1965). Very similar results have been obtained with young chickens, in which a different GVH assay method and several immunosuppres-

sant drugs were used (Floersheim and Seiler, 1967). The decrease in the GVH reaction when either donor cells or hosts are inactivated by CX or X-irradiation indicates that both are involved in the growth response as was reported earlier (Biggs and Payne, 1959; Howard et al., 1961; Jaffe and Fechheimer, 1966; Seto, 1967).

When prospective donors were exposed to cyclophosphamide as 17- to 18-day embryos, the development of the immune capacity was considerably delayed in the growing chicks. Apparently embryonic events necessary for the differentiation and proliferation of immunocyte precursors have been disrupted and several weeks are required for the regeneration of the depleted stem cell pool.

The effect of CX on the immune response is not specific and very similar to that reported for X-irradiation (Schwartz et al., 1957; Seto and Albright, 1965; Micklem ad Loutit, 1966; Glynn et al., 1968). The mechanism of the immunosuppressive action of CX is incompletely understood, but on the basis of known biochemical effects of alkylating agents (Wheeler, 1967) and their radiomimetic properties it is likely that many cell processes, including nucleic acid synthesis and DNA replication, are adversely affected. Cyclophosphamide produces its immunosuppressive effect by inhibiting cellular proceses and cell proliferation in lymphoid organs, but other sensitive organ systems are affected as well.

SUMMARY

The effects of cyclophosphamide (CX) on the GVH reaction in chick embryos and on the development of cellular immunity in baby chicks were investigated. The GVH competence of adult Game chicken blood decreased with exposure to increasing drug concentrations. A similar dose-response relationship was obtained when host embryos were treated with increasing amounts of CX prior to grafting of donor tissues. Marked reduction in the GVH reaction also occurred when CX treatment of host embryos was delayed 12 hr to 3 days after the inoculation of donor blood, indicating its susceptibility to immunosuppression up to three days. The GVH reaction capacity of White Leghorn donors, treated as embryos with CX, was much lover than that of untreated donors the first 6 weeks after hatching. The near-normal level of competence of 9-week-old, CX-treated donors indicates that considerable regeneration of the immune capacity has occurred. The findings indicate that treatment of chick embryos with sublethal doses of CX effectively suppresses the host component of the GVH reaction and delays the maturation of transplantation immunity.

TABLE I. EFFECT OF DELAYED EXPOSURE TO CYCLOPHOSPHAMIDE (CX) ON SPLENOMEGALY OF HOST EMBRYOS INOCULATED WITH GAME CHICKEN BLOOD.

Experiment	Mean spleen weights[1] (in mg)				
	Donor blood only	Time of CX treatment (in days)[2]			
		½	1	2	3
A	238	56	84	52	45
B	238	60	97	69	56

[1]Five to eight host embryos in a sample
[2]Time after inoculation of blood

LITERATURE CITED

Aisenberg, A. C., B. Wilkes, and B. H. Waksman. 1962. The production of runt disease in rats thymectomized at birth. J. Exp. Med. 116:759-772.

Barnes, B. A., P. B. Shad, and V. W. Pinn. 1966. Modification of the graft-versus-host reaction in F_1 mice treated with 6-mercaptopurine. Transplant. 4:154-158.

Biggs, P. M., and L. N. Payne. 1959. Cytological identification of proliferating donor cells in chick embryos injected with adult chick blood. Nature 184:1594.

Billingham, R. E. 1959. Reaction of grafts against their hosts. Sci. 130:947-953.

Floersheim, G. L. and K. Seiler. 1967. Differential effects of immunosuppressive drugs on a cutaneous graft-versus-host reaction in chickens. Transplant. 5:1355-1370.

Fox, Miles. 1964. Suppression of tissue immunity by cyclophosphamide. Transplant. 2:475-486.

Glynn, J. P., A. Fefer, and B. L. Halpern. 1968. Cyclophosphamide-induced chimerism. Cancer Res. 28:41-43.

Howard, J. G., D. Michie, and M. Simonsen. 1961. Splenomegaly as a host response in graft-versus-host disease. Brit. J. Exp. Pathol. 42:478-485.

Jaffe, W. P. and N. S. Fechheimer. 1966. The identification of cells during the proliferative phase of spleen enlargement of chick embryos following injection with adult blood. Poultry Sci. 45:269-271.

Karnofsky, D. A. 1968. Cancer chemotherapeutic agents. CA; Canc. J. Clin. 18:72-81.

Kryukova, I. N. 1959. The effect of an *in vitro* irradiation of spleen cells on induced splenomegaly in chick embryos. Folia Biol. 5:186-189.

Levy, L. 1966. The pharmacology of immunosuppressant drugs and experimental models. Surv. Ophthalmol. 11:444-453.

Micklen, H. S. and J. F. Loutit. 1966. *Tissue Grafting and Radiation.* Academic Press, New York. p. 1-228.

Owens, A. H. and G. W. Santos. 1968. The induction of graft versus host disease in mice treated with cyclophosphamide. J. Exp. Med. 128:277-291.

Santos, G. W. 1967. Immunosuppressive drugs I. Fed. Proc. 26:907-913.

Schwartz, E. E., A. C. Upton, and C. C. Congdon. 1957. A fatal reaction caused by implantation of adult parental spleen tissue in irradiated F_1 mice. Proc. Soc. Exp. Biol. Med. 96:797.

Seto, F. 1967. Graft-versus-host and host-versus-graft reactions in the chick embryo. Transplant. 5:1280-1288.

_____. 1968a. Quantitative study of the splenomegaly assay system of the graft-versus-host reaction in chick embryos. Proc. Okla. Acad. Sci. 47:134-139.

_____. 1968b. Variations in the graft-versus-host reaction (GVHR) capacity of growing chickens. Transplant. 6:771-782.

..... and J. F. Albright. 1965. An analysis of host and donor contributions to splenic enlargement in chick embryos inoculated with adult chicken spleen cells. Devel. Biol. 11:1-24.

Simonsen, M. 1957. The impact on the developing embryo and newborn animal of adult homologous cells. Acta. Pathol. Microbiol. Scand. 40:480-500.

Wheeler, G. P. 1967. Some biochemical effects of alkylating agents. Fed. Proc. 26:885-892.