Drug-Induced Immunologic Tolerance: Site of Action of Cyclophosphamide[1] (34558)

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Drug-induced immunologic tolerance is a selective deletion of immunologic responsiveness brought about by treatment with cytotoxic drugs (1). Mice can be rendered tolerant of sheep red blood cells (SRBC) by treatment with a single dose of the alkylating agent cyclophosphamide, and the tolerant state can be maintained for months if SRBC are injected periodically (2). Recent studies of this system demonstrated that antigen determines the specificity of tolerance and cyclophosphamide initiates its development by eliminating antigen-selected cells (3).

We now present evidence that the mechanism of induction of immunological tolerance in cyclophosphamide-treated mice is destruction by the drug of thymus-dependent lymphocytes. The immunological competence of thymocytes and bone marrow cells obtained from tolerant mice was analyzed by the method of Claman, Chaperon, and Triplett (4), who showed that cells from both the thymus and the marrow are essential for the immune response of the mouse to SRBC. The thymus-marrow interaction is synergistic rather than additive, and it appears that the marrow cell synthesizes antibody following an as yet undefined interaction with the thymocyte (5).

Materials and Methods. Animals. C57B1/6J mice, aged 8–10 weeks, were housed in plastic cages and had free access to water and a standard mouse diet (Purina).

Antigens. Sheep red blood cells (SRBC) (Colorado Serum Co.) and horse red blood cells (HRBC) (a gift of the Massachusetts Public Health Biological Laboratories) were washed three times in 0.15 *M* saline and resuspended to the desired concentration in 0.15 *M* saline.

Cyclophosphamide (Cytoxan, Mead Johnson Laboratories). The drug was dissolved in 0.10 *M* saline and administered intraperitoneally in a concentration of 3.0 mg/cc.

X-Irradiation. Mice were exposed in lucite containers to 850 R using a Westinghouse deep therapy unit (250 kV, 15 mA, target distance 50 cm) at a rate of 46 R/min.

Measurement of the immune response. The hemolytic plaque assay of Jerne and Nordin (6) was used. Serum hemagglutinins were estimated in microtiter plates (7). Titers are expressed as the number of the last well in the plate that showed an agglutination pattern.

Experimental Design. Tolerance of SRBC was induced in 8 to 10-week-old C57B1/6 mice by an intraperitoneal (ip) injection of 5×10^8 SRBC, followed 24 hr later by a single ip dose of cyclophosphamide, 150 mg/kg. Tolerance was maintained by ip injections of 5×10^8 SRBC every 5 days thereafter. Specificity of the tolerance was demonstrated by the normal response of these mice to HRBC given 5 days after treatment with cyclophosphamide (Table I).

The immunological competence of thymocytes and marrow cells from either tolerant or normal mice was tested after transfer of the cells to heavily X-irradiated recipients. Six groups of C57B1/6 mice were given 850 R total body X-irradiation. On the same day they received: (a) no other treatment (Group 1); (b) normal marrow cells (Group 2); (c) normal thymus cells (Group 3); (d) normal marrow cells and normal thymus cells (Group 4); (e) normal marrow cells and thymus cells from tolerant mice (Group

[1] Supported by U.S. Public Health Service Grant No. AM 07937.

TABLE I. Serum Hemagglutinin Titers in Normal and Tolerant Mice.[a]

Day	Control		Experimental	
	Anti-SRBC	Anti-HRBC	Anti-SRBC	Anti-HRBC
10	4.8	1.0	0.3	0.8
15	6.2	2.0	0.3	1.8
20	7.2	4.3	0.5	4.2
25	7.8	8.2	0.5	8.1
30	8.4	9.4	0.5	9.6
35	8.6	10.1	0.4	10.8

[a] Both groups received 5×10^8 SRBC on day 0, and every 5 days thereafter. On day 5 both groups received 5×10^8 HRBC and every 5 days thereafter. The experimental mice were given cyclophosphamide, 150 mg/kg on day 1. This rendered them specifically tolerant of SRBC. Twenty mice in each group.

5); and (f) marrow cells from tolerant mice and normal thymus cells (Group 6). In each case, the cell suspensions were given intravenously. Particular care was taken to avoid the parathymic lymph nodes when removing thymus tissue from the donor mice. The tolerant donors had been rendered tolerant of SRBC by treatment with cyclophosphamide, and had received at least four injections of 5×10^8 SRBC at 5-day intervals. At the time they were killed, these donors had no detectable antibodies against SRBC in their serum. All X-irradiated animals were challenged with 5×10^8 SRBC on the day of X-irradiation and 8 days later the number of hemolytic plaque forming cells in their spleens was enumerated.

Results. The results (Table II) confirm the finding of others (4, 5) that bone marrow and thymus cells act synergistically in restoring the immune response of X-irradiated mice. They also show that thymus cells from mice rendered tolerant of SRBC by cyclophosphamide treatment were ineffective in restoring immunity (Group 6). By contrast, marrow cells from tolerant animals were just as active as their counterparts from normal donors.

In the next group of experiments, mice rendered tolerant of SRBC were injected intravenously with either normal syngeneic marrow cells, normal syngeneic thymus cells, or a mixture of these two cells types. The "restoring" cells were administered 24 hr after cyclophosphamide treatment, and 3 days later 5×10^8 SRBC were given. Five days after this second challenge with SRBC, the animals were killed and the number of hemolytic plaque forming cells present in their spleens was determined. The results (Table III) indicate that immunological responsiveness was restored in the cyclophosphamide-treated mice by thymic cells, but not by bone marrow cells. By contrast with the results of restoration of X-irradiated ani-

TABLE II. Restoration of Heavily X-irradiated Mice with Thymocytes or Marrow Cells from Either Normal or Tolerant Mice.[a]

Group	Type of restoring cells	No. of restoring cells	Antibody forming cells/spleen
1	None	0	24 ± 7
2	Normal marrow	10^7	31 ± 8
3	Normal thymus	4×10^7	73 ± 14
4	Normal marrow and normal thymus	10^7 and 4×10^7	2178 ± 657
5	Tolerant marrow and normal thymus	10^7 and 4×10^7	2236 ± 692
6	Normal marrow and tolerant thymus	10^7 and 4×10^7	518 ± 124

[a] The number of antibody forming cells/spleen is expressed as the mean $\pm$ 1 SD. The $\log_{10}$ of the results was subjected to an analysis of variance: Group 4 vs. Group 5, $p > 0.2$; Groups 4 and 5 vs. Group 6, $p < 0.001$. Ten to 12 mice in each group.

TABLE III. Restoration of Cyclophosphamide-Treated Mice by Normal Thymocytes or Marrow Cells.[a]

Group	Type of restoring cells	No. of restoring cells	Antibody forming cells/spleen
1	None	—	572 ± 120
2	Marrow	2×10^7	1010 ± 275
3	Thymus	2×10^7	6192 ± 612
4	Marrow and thymus	2×10^7 and 2×10^7	6089 ±2920

[a] Results are given as the mean ± 1 SD. The data were subjected to an analysis of variance: Group 1 vs. Group 2, $p > 0.2$; Group 3 vs. Group 4, $p > 0.2$; Groups 1 and 2 vs. Groups 3 and 4, $p < 0.001$. Ten to 12 mice in each group.

mals, there was an apparent lack of thymus-marrow synergism (Group 4) in the cyclophosphamide-treated mice. This demonstration that thymocytes alone can fully restore immune competence in tolerant mice complements the results in Group 5 (Table II), which showed that the marrow of tolerant mice is immunologically intact.

A third group of experiments was carried out to determine if cyclophosphamide-induced tolerance involves macrophages or responsiveness to macrophage-processed antigen. Peritoneal exudates were induced by thioglycollate treatment (8) in normal and tolerant mice. Two hours before collection of the cells by paracentesis, 10^8 SRBC were injected intraperitoneally. Phagocytic cells containing the antigen were then harvested from the peritoneal cavity and 5×10^6 of them were injected ip into normal mice. Cells obtained from both tolerant and normal donors provoked similar immune responses in the test recipients. Macrophages containing SRBC were obtained from normal mice and injected into animals tolerant of SRBC, and no immune response was elicited. It would thus appear that cyclophosphamide treatment does not involve the mcarophage limb of the immune response, and that "processed" antigen cannot induce antibody synthesis in tolerant mice.

Discussion. The data shown in Tables II and III demonstrate that immunologic tolerance in cyclophosphamide-treated mice is effected at the level of the thymus or its exported cell, the thymus-dependent lymphocyte. Normal thymic cells restored responsiveness in tolerant mice, and thymocytes from tolerant mice failed to act synergistically with normal marrow cells in irradiated hosts. Assignment of the thymus-dependent lymphocyte as a site of action of cyclophosphamide is consistent with the observation that thymectomy greatly enhances the immunosuppressive effects of this drug (9, 10). These results indicate that thymocytes have an antigen-specific function in antibody synthesis, since the tolerant mice are unresponsive only to those antigens present on the tolerizing SRBC. We suggest that SRBC activate an antigen-reactive line of thymus-dependent cells, thereby making them differentially susceptible to the cytotoxic drug.

Our results are in accord with those of Taylor (11), who found that thymus cells, but not marrow cells, were paralyzed 24 hr after the administration of a large amount of bovine serum albumin to mice. The data we obtained are, however, in direct opposition to those of Playfair (12), who reported that the marrow cells of cyclophosphamide-treated mice are immunologically incompetent, whereas their thymocytes behave normally. The reason for these discordant results is presently unknown. Apart from methodological differences between the experiments (*e.g.*, we selected for study only those animals with solid, established tolerance; the tolerance in Playfair's mice was only transient), it may be of interest that the animals used by Playfair were (NZB × Balb)F_1. The NZB mouse seems to have an unusual tolerance mechanism (13), perhaps resulting from a thymic lesion (14). No such disorder

is known to occur in the mice we studied (C57B1/6). It should be possible to explore these differences with comparative studies in different strains of mice.

Since immunosuppression by antilymphocyte serum is also due to effects on thymus-dependent lymphocytes (15), it is conceivable that this line of cells is the target for a variety of chemical and biological immunosuppressants. Finally, since the marrow is immunologically normal in drug-induced immunologic tolerance, myelosuppression may be unnecessary for effective and specific immunosuppression.

Summary. The cellular mechanism of immunologic tolerance in cyclophosphamide-treated mice was analyzed. Thymocytes from these animals failed to act synergistically with normal marrow cells in heavily irradiated recipients. By contrast, the marrow cells of tolerant mice cooperated with normal thymocytes in the X-rayed recipients. Tolerance in the drug-treated mice was broken by administration of thymocytes, but not by marrow cells. It was concluded that the target cell in drug-induced immunologic tolerance is a thymus-dependent lymphocyte.

1. Schwartz, R. S., Fed. Proc. **25**, 165 (1966).

2. Aisenberg, A. C., J. Exp. Med. **125**, 833 (1967).

3. Many, A., and Schwartz, R. S., Clin. Exp. Immunol. **6** 1970.

4. Claman, H. N., Chaperon, E. A., and Triplett, R. F., Proc. Soc. Exp. Biol. Med. **122**, 1167 (1966).

5. Mitchell, G. F., and Miller, J. F. A. P., J. Exp. Med. **128**, 821, (1968).

6. Jerne, N. K., and Nordin, A. A., Science **140**, 405 (1963).

7. Sever, J. L., J. Immunol. **88**, 320 (1962).

8. Morita, T., and Perkins, E. H., J. Reticuloendothel. Soc. **1**, 406 (1964).

9. Dukor, P., and Dietrich, F. M., Int. Arch. Allergy **32**, 131 (1967).

10. Aisenberg, A. C., and Davis, C., J. Exp. Med. **128**, 35 (1968).

11. Taylor, P., Nature **220**, 611 (1968).

12. Playfair, J. H. L., Nature **222**, 282 (1969).

13. Staples, P. J., and Talal, N., J. Exp. Med. **129**, 123 (1969).

14. DeVries, M. J., and Hijmans, W., Immunology **12**, 179 (1967).

15. Martin, W. J., and Miller, J. F. A. P., J. Exp. Med. **128**, 855 (1968).

Received Oct. 13, 1969. P.S.E.B.M., 1970, Vol. 133.