IMMUNOLOGIC TOLERANCE PRODUCED IN ADULT ANIMALS BY COMBINED INJECTION OF CYCLOPHOSPHAMIDE AND ANTIGEN (SHEEP'S ERYTHROCYTES)

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As a result of combined injections of antigen and cyclophosphamide into adult mice, the recipients develop immunologic tolerance to this antigen. The animal's lymphocytes become incapable of reacting to this antigen either in situ or when cultivated in vivo. The ability to develop an immunologic response to a different antigen is retained.

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The first attempts to produce immunologic tolerance in adult animals by combined administration of antigen and immunodepressant were made 11 years ago [20]. Later as a result of several failures [4,5,16], interest in this problem diminished, and it has increased again only recently in the course of a search for new and more effective immunodepressant agents [6, 10, 18]. The growth of interest in the problem of "drug-induced tolerance" was stimulated by the fact that other methods of production of tolerance or of allied states (immunologic paralysis, the "overloading" phenomenon, Dresser's phenomenon) in adult animals are applicable only to a limited range of antigens and make high demands on their quality.

The object of the investigation described below was to produce tolerance against sheep's erythrocytes in adult mice by combined injection of the antigen and of cyclophosphamide*, a highly effective immunodepressant [2, 7, 12, 15, 19].

EXPERIMENTAL METHOD

Experiments were carried out on adult (18-30 g) male CC57BR mice, on F_1 (CBA × C57BL) hybrid mice, and on noninbred albino mice. The experimental mice received an intraperitoneal injection of $6 \cdot 10^9$-$6.2 \cdot 10^9$ sheep's erythrocytes in a volume of 0.5 ml. After an interval of 42-48 h, the same mice received cyclophosphamide intraperitoneally in a dose of 200 mg/kg. Control animals received either cyclophosphamide alone or antigen alone, or were untreated. All the mice (including the controls) received an intravenous injection of a test dose of antigen ($5 \cdot 10^8$ sheep's erythrocytes) after 1-3 weeks. In some experiments, carried out to test the specificity of tolerance, instead of sheep's erythrocytes, the same dose of rat's erythrocytes was injected.

The animals were sacrificed 4 days after the test injection of antigen. The number of antibody-forming cells in the spleen was studied by the local hemolysis in gel test [11]. This reaction was carried out with the same antigen as was used for the test injection. Some details of the method used in this test have been described previously [1, 3]. The titer of antibodies in the blood serum (hemagglutinins and hemolysins) was also tested, starting with a dilution of 1:10.

In some experiments the method of cultivation of spleen cells in vitro was used. Recipient mice, irradiated with γ-rays in a dose of 600-700 R, were injected intravenously with $1 \cdot 10^8$ donors' spleen cells,

*Synonyms: endoxan, cytoxan.

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TABLE 1. Induction of Immunologic Tolerance in Adult Animals by Combined Injections of Antigen and Cyclophosphamide

Preliminary injections of	Day of injection of test dose of antigen	Number of mice	Number of nucleated cells in spleen ($\times 10^6$)	Number of antibody-forming cells in spleen and confidence limits ($\times 10^3$)	Distribution of mice according to number of antibody-forming cells in spleen		
					$<10^3$	10^3–10^4	$>10^4$
6.2×10^9 erythrocytes, followed 2 days later by cyclophosphamide	7	72	449	<1	42	20	10
	21	24	324	8 (3—17)	2	14	8
Cyclophosphamide	7	68	502	34 (27—43)	—	10	58
	21	21	323	104 (85—127)	—	—	21
6.2×10^9 erythrocytes	7	21	403	33 (19—59)	—	5	16
	21	8	377	97 (56—169)	—	—	8
—	7	63	428	156 (131—184)	—	—	63
	21	18	375	204 (165—252)	—	—	18

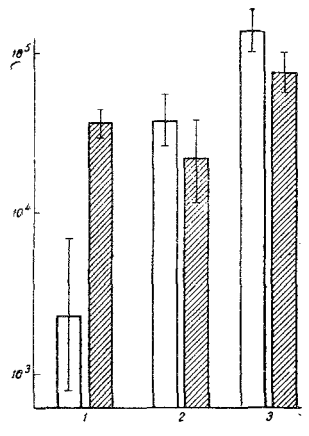

Fig. 1. Specificity of tolerance induced by combined injection of antigen and cyclophosphamide: 1) mice receiving injections of sheep's erythrocytes and cyclophosphamide; 2) mice receiving cyclophosphamide only; 3) intact mice. Unshaded columns represent number of antibody-forming cells in spleen after test injection of sheep's erythrocytes; shaded columns show number of antibody-forming cells after test injection of rat's erythrocytes. Ordinate: number of antibody-forming cells.

suspended in medium No. 199. A test dose of sheep's erythrocytes was injected intravenously into the recipients 1-2 h later. The recipients were sacrificed after 5 days and their spleen investigated by the local hemolysis test. Either animals in which tolerance had previously been created (as indicated above) or control animals were used as donors and recipients in the different series of experiments. The experiments were carried out in an isolinear system.

All the results were analyzed by statistical methods (the geometric mean and confidence limits for $P < 0.05$ were calculated).

EXPERIMENTAL RESULTS

Changes in immunologic reactivity resulting from injection of a large dose of antigen and of cyclophosphamide separately, and also from their combined administration were investigated to begin with. The results are given in Table 1, and they show that preceding injections of cyclophosphamide and of a large dose of antigen separately produced some depression of immunologic reactivity, most marked when tested after one week. However, the combined injection of antigen with cyclophosphamide gave an incomparably greater effect: under these conditions the immunologic reactivity of the animals was reduced by 1 or 2 orders of magnitude compared with the control. When immunologic reactivity of the animals was tested one week after combined injections of antigen and cyclophosphamide, the immune response was completely or almost completely suppressed in most animals (in 42 of 72). Immunologic reactivity three weeks later was partly restored, although still remaining much below that in the animals of the control groups.

Similar results were obtained when the antibody titers were investigated. Hemolysins were absent (< 1 : 10) in the blood sera of mice receiving the test dose of antigen 7 days after combined injections of antigen and cyclophosphamide, while titers of hemagglutinins were sharply reduced (1 : 20-1 : 30) compared with control groups (1 : 320-1 : 640).

In the next series of experiments, carried out on 132 mice, the specificity of the observed effect was investigated (Fig. 1).

Combined injections of sheep's erythrocytes and cyclophosphamide induced depression of immunologic reactivity against the homologous antigen only (Fig. 1). The immune response of the experimental animals

TABLE 2. Crossed Transplantation of Spleen Cells of Tolerant and Intact Mice

Scheme of experiments	Number of recipients	Number of nucleated cells in spleen ($\times 10^6$)	Mean number of antibody-forming cells and confidence limits
T → I	10	89	<35 (<53)
CP → I	10	112	176 (62-503)
I → I	10	54	2,147 (1,372-3,361)
I → T	10	130	2,393 (1,581-3,624)
I	8	14	<8

Legend: T) Mice receiving cyclophosphamide preceded by antigen; CP) mice receiving cyclophosphamide only; I) intact mice. All recipients preliminarily irradiated in dose of 600-700 R. Test dose of antigen injected into animals after transplantation of cells.

(group 1) to foreign antigen (rat's erythrocytes) was no weaker than in animals receiving preliminary injections of cyclophosphamide only (group 2). Similar results have been obtained by other works [8, 17].

The object of the next experiments was to determine whether combined injection of antigen and cyclophosphamide produces a true deficiency of cells competent with respect to that particular antigen or whether the observed changes in immunologic reactivity are due to other factors. Spleen cells from mice previously receiving combined injections of antigen and cyclophosphamide were transplanted into irradiated, intact mice. The complete scheme of the experiments and their results are given in Table 2. Spleen cells of mice receiving preliminary combined injection of antigen and cyclophosphamide gave a much weaker reaction to antigen in the recipients than cells of control animals. Conversely, cells of intact mice reacted equally well to antigen both in intact recipients and in recipients receiving preliminary injection of antigen together with cyclophosphamide.

The results of these experiments suggest that as a result of the combined administration of antigen and cyclophosphamide, cell clones competent with respect to that antigen are eliminated from the general population of lymphocytes. The state which thus arises corresponds to the principal criteria of immunologic tolerance [13].

With the course of time, tolerance evoked by combined injections of antigen and cyclophosphamide disappears spontaneously. However, it was not replaced by the opposite state of sensitization to the same antigen, as has been reported by some workers [9, 14] in the "overloading" phenomenon.

In conclusion, it must be stressed that tolerance can be induced in adult animals to a "strong" corpuscular antigen by combined injections of the antigen and cyclophosphamide. It is known that tolerance to such antigens is difficult to induce, even in newborn animals. It may therefore be hoped that this method will prove useful in the future for inducing tolerance in the postnatal period to weaker transplantation antigens.

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