MECHANISM OF TOLERANCE TO *Salmonella typhi* Vi ANTIGEN INDUCED WITH CYCLOPHOSPHAMIDE

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Treatment with Vi antigen followed after 46-48 h by cyclophosphamide induces a state of specific areactivity in mice which persists through adoptive transfer. Only trace amounts of Vi antigen were found in the blood and spleen of the tolerant mice after 2-3 weeks. No T suppressors were found in the spleen of the tolerant animals: Cells of the tolerant mice did not depress the immune response of normal lymphocytes when cultured together *in vivo* and they did not induce tolerance in intact recipients; the cells of normal donors partially restored the immunoreactivity of the tolerant animals. The results suggest that this form of tolerance is due to elimination or prolonged inactivation of the immunocompetent cells.

KEY WORDS: *tolerance; Vi antigen; cyclophosphamide.*

One way of inducing immunological areactivity is by injecting the immunodepressant cyclophosphamide at the peak of proliferation of the immunocompetent cells in response to a supraimmunogenic dose of an antigen. This state of areactivity is generally called cyclophosphamide tolerance. It has been well studied in the case of areactivity to sheep's red cells: thymus-dependent corpuscular antigen [5-7, 12, 13]. Doubts have been expressed about the possibility of obtaining tolerance of this type to polysaccharide antigens, for large doses of polysaccharides induce immunological paralysis by blocking receptors on the surface of the lymphocytes [11]. Nevertheless, cyclophosphamide tolerance to a high-molecular-weight polysaccharide antigen, namely the Vi antigen of *Salmonella typhi*, has been obtained in the writers' laboratory [6].

The object of this investigation was to study the mechanism of this form of immunological areactivity to Vi antigen.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred mice and on (CBA × C57BL/6)F_1 male mice weighing 18-20 g. A commercial preparation of *Salmonella typhi* Vi antigen produced by the Moscow Scientific-Research Institute of Epidemiology and Microbiology [1] was used as the antigen. To induce tolerance the mice were given an intravenous injection of 200 μg Vi antigen followed after 46-48 h by an intraperitoneal injection of cyclophosphamide in a dose of 200 mg/kg. The experimental and control animals were given an intravenous injection of 10 μg of Vi antigen 2-3 weeks later. The number of antibody-forming cells (AFC) in the spleen was counted by the passive hemolysis in gel method using sheep's red cells loaded with Vi antigen [2].

In adoptive transfer experiments tolerant donors 2-3 weeks after the induction of tolerance and syngeneic recipients irradiated on a cobalt source in a dose of 700 rad were used. The recipients were given an intravenous injection of $5 \cdot 10^7 - 1 \cdot 10^8$ spleen cells of the ex-

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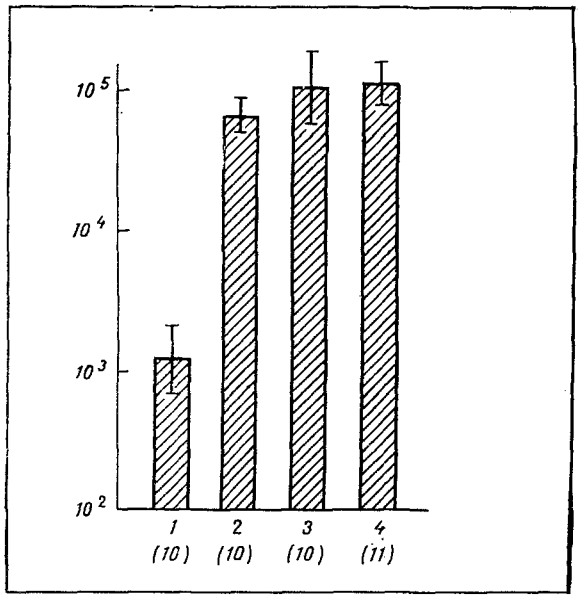

Fig. 1

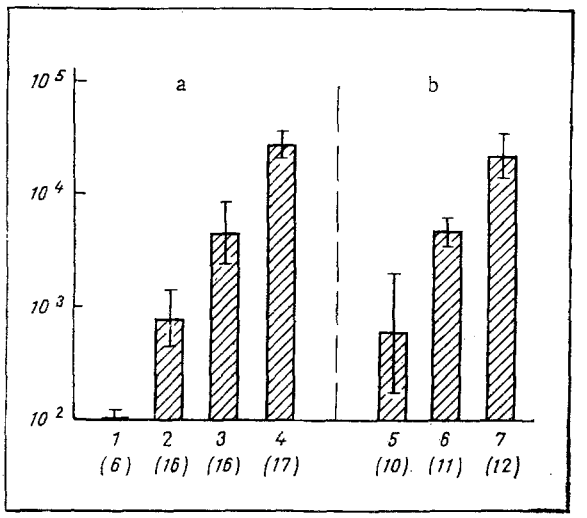

Fig. 2

Fig. 1. Decrease in immunoreactivity of mice 2-3 weeks after combined injection of Vi antigen and cyclophosphamide. 1) Preliminary treatment of mice with 200 μg Vi antigen and cyclophosphamide; 2) cyclophosphamide; 3) 200 μg Vi antigen; 4) intact mice. Number of animals in group shown in parentheses. Ordinate, number of AFC in spleen of mice on fourth day after injection of test dose of antigen.

Fig. 2. Immune response of spleen cells of tolerant donors in adoptive transfer (a) and combined culture *in vivo* with spleen cells of intact donors (b). Groups of animals: a) mice irradiated in a dose of 700 rad (1), irradiated with 700 rad and receiving 10^8 spleen cells of tolerant donors (2), of donors treated with cyclophosphamide (3), and of intact donors (4); b) recipients of $5 \cdot 10^7$ spleen cells of tolerant (5) and intact (6) donors of $5 \cdot 10^7$ of both types of cells (7). Number of animals in group shown in parentheses. Ordinate, number of AFC in recipients' spleen on fifth day after injection of donors' cells and of antigen.

perimental donors mixed with 10 μg Vi antigen 1-3 h after irradiation. The number of AFC in the recipient's spleen was counted 5 days later.

To determine the concentrations of Vi antigen in the blood serum of the tolerant animals the passive hemagglutination inhibition test (PHIT) was used [4] with the following modifications: Sheep's red cells sensitized for 45 min at 37°C with 10 μg Vi antigen were used and the reaction was carried out in a phosphate buffer, pH 7.2. Student's criterion was used in the statistical analysis.

EXPERIMENTAL RESULTS

Tolerogenic treatment sharply reduced (by 50-100 times) the ability of the animals to give a specific immune response. Injection of Vi antigen and cyclophosphamide separately gave no effect (Fig. 1).

The following possible mechanisms of tolerance to Vi antigen were investigated: reversible blockage of lymphocytes by the antigen, activation of T suppressors, or a deficiency of immunocompetent cells.

In the first series of experiments the quantity of antigen in the blood serum and in the spleen of the tolerant animals was investigated. Two weeks after the induction of tolerance, either no Vi antigen was present in the blood serum of the mice, judging from the results of the PHIT, or it was present in only very small amounts, not more than 0.35 μg/ml. The spleen of the tolerant mice likewise did not contain any substantial amount of antigen: Transfer of 10^8 spleen cells of tolerant donors to intact mice without simultaneous injection of the antigen did not induce an immune response in the recipients.

TABLE 1. Partial Restoration of Immunoreactivity of Tolerant Animals after Injection of Spleen Cells from Intact Donors

Cells transferred	Recipients	Number of mice	Mean number of AFC per spleen*
—	Tolerant	13	807 (386—1687)
—	Intact	11	111 780 (78796—158308)
10^8 spleen cells from intact donors	Tolerant	17	5049 (3300—7717)
	Tolerant, irradiated with 700 rad	17	7886 (3529—17632)
10^8 spleen cells from tolerant donors	Tolerant	7	1305 (800—2132)
	Intact	16	76 094 (52623—109856)

*Confidence limits shown in parentheses.

The state of areactivity persisted through adoptive transfer of spleen cells of tolerant donors into irradiated recipients (Fig. 2a) and, consequently, it was not due to the reversible blockade by the antigen of the receptors of antigen-recognizing or antibody-forming cells.

These facts as a whole do not confirm the hypothesis of paralysis by excess of antigen and of reversible blockade of the lymphocytes in tolerance to Vi antigen induced with the aid of cyclophosphamide.

The object of the next series of experiments was to discover whether the form of areactivity discovered is due to a true deficiency of immunocompetent cells or to activation of T suppressors [8-10]. As is clear from Fig. 2b, during combined culture *in vivo* the spleen cells of tolerant donors not only did not depress the immune response of intact donors, but actually enhanced it.

Another series of experiments showed that transferring spleen cells from intact donors into tolerant mice enhanced the immune response of the recipients by five to six times, i.e., led to partial loss of tolerance (Table 1). The reason why recovery of immunoreactivity was incomplete was evidently the insufficient number (10^8) of immunocompetent cells injected. In fact, 10^8 spleen cells from normal animals, even if injected into irradiated tolerant mice, induced an immune response of the same order (7886 AFC per spleen). Injection of spleen cells of tolerant animals into intact syngeneic recipients did not affect the immunoreactivity of the latter.

No signs of "infectiousness" of tolerance or of its resistance to breaking down by normal cells could thus be detected. The results described above suggest that tolerance to Vi antigen of *S. typhi*, induced in adult mice with the aid of cyclophosphamide, is due to elimination or irreversible blockade of immunocompetent cells. Reversible blockade of cellular antigen receptors or activation of T suppressors evidently had no decisive role in this phenomenon.

Comparison of the results of these experiments with data in the literature [6, 9] emphasizes the considerable similarity between this form of areactivity and the tolerance induced under similar conditions toward sheep's red cells: In both cases T suppressors are absent and the principal mechanism of areactivity is elimination or irreversible inactivation of the immunocompetent cells.

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