CONDITIONS OF FORMATION AND PROLONGATION OF THE STATE OF IMMUNOLOGIC TOLERANCE INDUCED IN ADULT ANIMALS BY COMBINED INJECTIONS OF ANTIGEN AND CYCLOPHOSPHAMIDE

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The importance of dose of antigen and time of administration of cyclophosphamide as conditions of formation of tolerance in adult animals was studied. It was shown that a state of tolerance can be prolonged by means of additional injections of antigen. Preliminary sensitization of the animals with a small dose of antigen prevented the formation of tolerance.

* * *

After administration of cyclophosphamide, adult animals for some time partially or totally lose their ability to react to an antigen by antibody formation [3, 6, 14, 19, et al.]. If injection of cyclophosphamide is preceded by an antigen, the state of immunodepression lasts longer [4, 5] and becomes specific in character [4, 9, 18]. The state thus arising corresponds to the basic features of immunologic tolerance [4].

The object of this investigation was to study the conditions of formation and prolongation of tolerance induced by combined injections of antigen and cyclophosphamide.

EXPERIMENTAL METHOD

Experiments were carried out on adult male CC57BR mice and on noninbred albino mice. Sheep's erythrocytes were injected in various doses (in most experiments 6×10^9 cells, intraperitoneally) to produce tolerance. Cyclophosphamide was injected intraperitoneally as a single dose of 200 mg/kg, as a rule 42-48 h after injection of the antigen. Control animals received either antigen alone or cyclophosphamide alone, or remained untreated. After various intervals (in most experiments, 7 days) all animals (including the controls) received an intravenous injection of a test dose of antigen (5×10^8 erythrocytes), and 4 days later the number of antibody-forming cells in the spleen was determined by the method of local hemolysis in gel [2, 13]. The results obtained were treated by statistical methods.

EXPERIMENTAL RESULTS

To begin with, the importance of dose of antigen and mode of its administration for the formation of tolerance was investigated. Two days before the injection of cyclophosphamide, the experimental mice were injected intravenously or intraperitoneally with different doses of erythrocytes (5×10^7, 5×10^8, or 4×10^9). A test dose of antigen was injected 7 days after the injection of cyclophosphamide.

It is clear from Fig. 1 that tolerance was formed only if the injection of cyclophosphamide was preceded by injection of the highest dose of antigen (4×10^9 erythrocytes). When smaller doses were given, the immunologic reactivity of the animals was either identical with that of animals receiving cyclophosphamide alone, or actually exceeded it. Evidently, with the intraperitoneal method of injection of antigen, the conditions are more favorable for development of tolerance than if the intravenous method is used, but the available experimental evidence is insufficient for a final solution of this problem.

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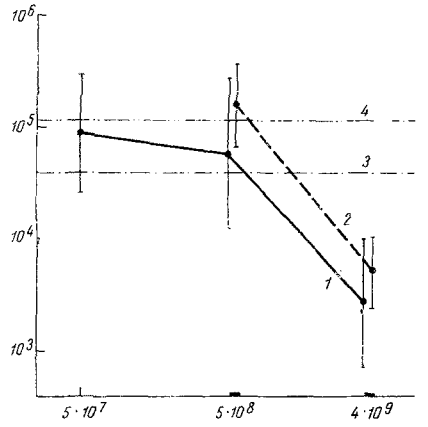

Fig. 1

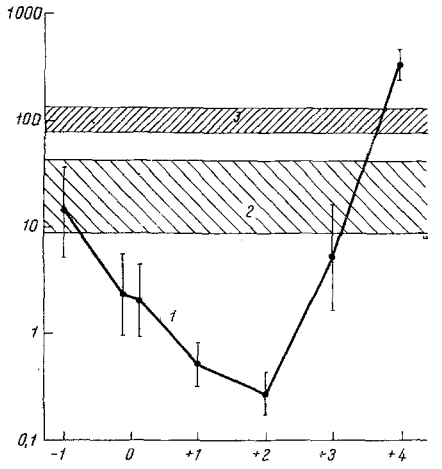

Fig. 2

Fig. 1. Importance of dose of antigen and mode of its administration for induction of tolerance. Ordinate, number of antibody-forming cells in spleen 4 days after test injection of antigen (5×10^8 sheep's erythrocytes); abscissa, dose of antigen injected 2 days before cyclophosphamide; 1) mice receiving injection of antigen intraperitoneally + cyclophosphamide; 2) mice receiving injection of antigen intravenously + cyclophosphamide; 3) mice receiving cyclophosphamide only; 4) control. Test injection of antigen given 7 days after injection of cyclophosphamide.

Fig. 2. Relationship between development of tolerance and times of injection of cyclophosphamide. Ordinate, number of antibody-forming cells in spleen after test injection of antigen, given 7 days after injection of cyclophosphamide (in percent of control); abscissa, days of injection of cyclophosphamide before (-) or after (+) tolerance-inducing injection of antigen; 1) mice receiving combined injections of antigen and cyclophosphamide; 2) mice receiving preliminary injections either of antigen only or of cyclophosphamide only; 3) control (intact mice receiving test injection of antigen).

In the next series of experiments the role of persistence of antigen in the maintenance of tolerance was studied. The experimental mice were injected intraperitoneally with 6×10^9 sheep's erythrocytes, followed 2 days later by cyclophosphamide. Some of them received additional injections of antigen in the same dose twice (on the 7th and 14th days after the first injection). On the 23rd day after the beginning of the experiment, when under normal conditions tolerance is partly lost [4], all the animals (including the controls) received the additional injections of antigen, but this time tolerance was considerably reduced.

In the experiments of series III the optimal times between injections of antigen and cyclophosphamide were determined. The mice (197) received cyclophosphamide either before injection of the antigen (24, 12, or 3 h) or after injection (3 h, 1, 2, or 3 days). The antigen was injected intraperitoneally in a dose of 6×10^9 erythrocytes. On the 7th day after injection of cyclophosphamide all the animals were injected with a test dose of antigen.

Tolerance was most marked if cyclophosphamide was injected 2 days after the antigen (Fig. 2). The closer the injections of erythrocytes and cyclophosphamide, the less the tolerance-inducing effect, although it was manifested even if both injections were given on the same day (3 h before or 3 h after injection of antigen). If the intervals between injections exceeded 2 days, this effect disappeared, and was sometimes replaced by the opposite state – increased immunologic reactivity toward the particular antigen (4 days after injection of antigen).

In the experiments of series IV the possibility of producing tolerance in animals previously sensitized with this particular antigen was studied. Mice (42) were injected intravenously with 1×10^6 sheep's erythrocytes. These animals, and also a group of intact animals, were injected intraperitoneally 7-16 days later with 6×10^9 sheep's erythrocytes, after 42-48 h with cyclophosphamide, and again after 7 days with a test dose of antigen.

TABLE 1. Prolongation of State of Partial Immunologic Tolerance by Repeated Injections of Antigen

Treatment of animals before test injection of antigen*	Number of mice	Distribution of mice according to number of antibody-forming cells in spleen			Mean number of antibody-forming cells in spleen ($\times 10^3$)	Confidence interval ($\times 10^3$)	P
		$< 10^3$	10^3–10^4	$> 10^4$			
Antigen + cyclophosphamide + additional injections of antigen	17	6	5	6	4	1–15	
Antigen + cyclophosphamide	14	–	7	7	20	6–62	< 0.05
Cyclophosphamide . .	11	–	–	11	99	75–131	< 0.003
Control	9	–	–	9	203	150–274	< 0.002

*Explanation in text.

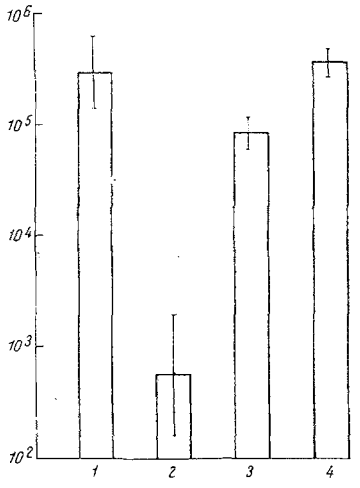

Fig. 3. Resistance of sensitized animals to induction of immunologic tolerance. Ordinate, number of antibody-forming cells in spleen 4 days after test injection of antigen; 1) sensitized mice receiving tolerance-inducing injection of antigen + cyclophosphamide; 2) intact mice receiving tolerance-inducing injection of antigen + cyclophosphamide; 3) mice receiving cyclophosphamide; 4) control.

As Fig. 3 shows, only the mice of the second group developed tolerance. In the mice of group 1 (preliminarily sensitized with a small dose of antigen) no tolerance developed under identical conditions. On the contrary, their immunologic reactivity was actually higher than that of the mice of group 3, receiving cyclophosphamide alone.

During the analysis of these results it must first be remembered that the optimal situation for tolerance formation is that in which the immunologic depressants act when the process of immunization has just begun. The principal object against which the action of cyclophosphamide or its metabolic products [1, 7] is directed is then,evidently, not the initial phase of the immune response (seizure of the antigen by macrophages and their interaction with lymphocytes), as is assumed in the case of administration of some other immunodepressants [11, 15, 17], but its later phase – the phase of proliferation of antibody-forming cells and their precursors. The productive phase of the immune response of mice to sheep's erythrocytes is known [2, 20] to begin during the first 1-2 days, and it is at this stage that the injection of cyclophosphamide produced the most marked tolerance (Fig. 2). It should be noted that the immunodepressant action of cyclophosphamide was also most marked when the compound was injected on the day of immunization or 2 days after injection of the antigen [3]. Later, in the state of completed differentiation of antibody-forming cells (3-4 days after injection of antigen), cyclophosphamide was ineffective.

Hence, as a result of the successive action of antigen and cyclophosphamide on the population of lymphoid cells, a selective elimination of cell clones involved in the immune response may be considered to take place. From this point of view it is easy to understand the need for giving large doses of antigen, sufficient to ensure involvement not only of regional, but also of distant lymphoid organs in the immune response.

Special experiments undertaken by the writers in fact showed that after intraperitoneal injection of 6×10^9 sheep's erythrocytes into mice, a large number of antibody-forming cells is found not only in the spleen, but also in distant (cervical) lymph glands. After injection of smaller doses of antigen, antibody formation was absent in the distant lymph glands.

It may also be considered that when large doses of antigen are used, the antigen persists longer in the body, thus enabling complete blocking of the immune response by cyclophosphamide. Under these circumstances a situation may arise which is similar to the conditions of tolerance formation in the immunologically immature organism: contact between the persistent antigen and the newly formed immature immunocompetent cells. Support for this view is given by the facts described above, namely, that tolerance induced by successive injections of antigen and cyclophosphamide can be prolonged by additional injections of antigen. Similar facts are known in relation to tolerance formed during the period of immunologic immaturity [12, 16].

Resistance of sensitized mice to the induction of immunologic tolerance discovered by these experiments suggests that this form of tolerance differs from the phenomenon of overloading, which can be induced in preliminarily immunized animals [8, 10].

LITERATURE CITED

1. A. A. Zidermane et al., in: Cyclophosphamide [in Russian], Riga (1965), p. 85.
2. L. A. Pevnitskii et al., Byull. Éksperim. Biol. i Med., No. 10, 60 (1967).
3. L. A. Pevnitskii et al., Byull. Éksperim. Biol. i Med., No. 10, 59 (1969).
4. L. N. Fontalin et al., Byull. Éksperim. Biol. i Med., No. 11, 60 (1969).
5. A. C. Aisenberg and C. Davis, J. Exp. Med., 128, 35 (1968).
6. M. C. Berenbaum and I. N. Brown, Immunology, 7, 65 (1964).
7. N. Brook and H.-J. Hohorst, Arzneimittel-Forsch., 13, 1021 (1963).
8. H. N. Claman and E. A. Bronsky, J. Allergy, 38, 208 (1966).
9. F. M. Dietrich and P. Dukor, Path. Microbiol. (Basel), 30, 909 (1967).
10. M. M. Dorner and J. W. Uhr, J. Exp. Med., 120, 435 (1964).
11. R. Gallily and M. Feldman, Immunology, 12, 197 (1967).
12. M. Hasek and A. B. Puza, in: Mechanism of Immunological Tolerance, Prague (1962), p. 257.
13. N. K. Jerne and A. A. Nordin, Science, 140, 405 (1963).
14. H. I. Maibach and H. C. Maquire, Internat. Arch. Allergy, 29, 209 (1966).
15. D. Nachtigal et al., Immunology, 15, 343 (1968).
16. G. J. V. Nossal et al., in: Mechanisms of Immunological Tolerance, Prague (1962), p. 151.
17. J. F. Pribnow and M. S. Silverman, J. Immunol., 98, 225 (1967).
18. S. B. Salvin and R. F. Smith, J. Exp. Med., 119, 851 (1964).
19. G. W. Santos and A. H. Owens, Bull. Johns Hopk. Hosp., 114, 384 (1964).
20. J. Sterzl et al., in: Molecular and Cellular Basis of Antibody Formation, Prague (1965), p. 463.