INVESTIGATION OF THE PHASES OF IMMUNOLOGICAL TOLERANCE INDUCED IN ADULT MICE BY MEANS OF CYCLOPHOSPHAMIDE

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Tolerance to sheep's red cells induced in adult mice by means of cyclophosphamide was investigated. The animals exhibited partial immunolgical areactivity for 3 months. In the first 2 weeks an antigen capable of inducing a secondary response to its action on the lymphoid cells of animals sensitized with sheep's red cells was present in the mice. After 3-4 weeks antibodies detectable by means of antiglobulin serum and capable of weakening the immune response of intact lymphoid cells injected into tolerant recipients appeared in the blood of the partially tolerant animals. The serum of tolerant animals inhibited the immune response of intact mice immunized with sheep's red cells. Meanwhile the lymphoid cells of tolerant mice preserved their immunological areactivity in culture in vivo. It is concluded that after the combined injection of antigen and cyclophosphamide a state of "true" tolerance appeared intially in the animals, and this was gradually replaced by autoregulation of the immune response of the feedback type. Both phenomena may coexist for a time and mutually assist each other.

The writers have shown previously [3] that the tolerance which arises in mice after the combined injection of sheep's red blood cells (SRBC) and cyclophosphamide (CP) satisfies the basic demands of true immunological tolerance [15]. It was shown, in particular, that during the two weeks after induction, tolerance is due to a deficiency of immunocompetent cells. According to recent reports in tolerance to serum proteins and to transplantation antigens produced in adult and newborn mice, the specific lowering of immunoreactivity is due not only to a decrease in the number of immunocomptent cells, but also to humoral agents (possible antibodies) formed in the tolerant animals which have a depressive action [8, 12, 19-21].

The object of the present investigation was to determine whether the deficiency of immunocompetent cells is the sole factor which determines tolerance to SRBC in mice at various times after its induction by means of CP.

EXPERIMENTAL METHOD

Experiments were carried out on male nonimbred and (CBA x C57BL/6)F_1 hybrid albino mice weighing initially 18-23g. Tolerance to SRBC was induced by the scheme described previously [3]: intraperitoneal injection of 200 mg/kg CP. Intact mice and mice receiving CP only acted as the controls. The immunoreactivity of the animals was estimated from the number of antibody-forming cells (AFC)

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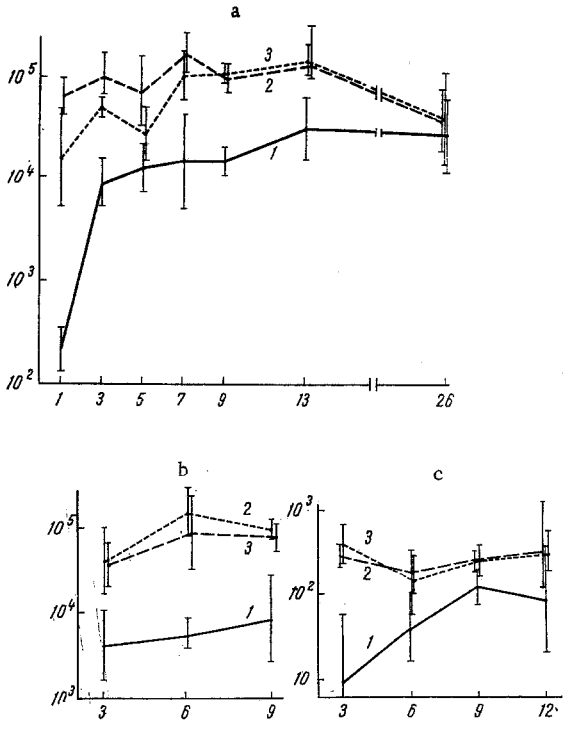

Fig. 1. Duration of tolerance in mice after combined treatment with SRBC and CP: a) 19S AFC in the spleen; b) 7S AFC in the spleen; c) 19S AFC in the lymph glands. Ordinate: a and b) number of AFC in the spleen; c) number of AFC per million nucleated lymph gland cells. Abscissa: time of test injection of SRBC after induction of tolerance (in weeks). Immune reaction of animals to test injection of SRBC: a) tolerant; 2) receiving CP only; 3) intact.

in the spleen after an intravenous test injection of 5×10^8 SRBC. The determination was made by Jerne's method [13] on the 4th day after the test injection (detection of "direct" plaques, or 19S AFC) and also on the 10th day using rabbit antiserum against mouse γ-globulins ("indirect" plaques, or 7S AFC [10, 18]). In some experiments the number of 19S AFC in the cervical lymph glands was investigated on the 5th day after intralabial injection of the test dose of antigen. Hemolysins and hemagglutinins were determined in the blood of the animals by the usual method of double serial dilutions. In some cases the titration was carried out by adding antiglobulin serum to the system of test serum + SRBC.

The immunoreactivity of the spleen cells in culture in vivo was determined by the method described by the writers previously [3]. In some experiments the donors were sensitized by the preliminary intravenous injection of 1×10^6 SRBC.

EXPERIMENTAL RESULTS

The duration of the tolerance arising after treatment of the animals by the scheme used was investigated in the experiments of series 1, which were conducted on 336 noninbred albino mice (Fig. 1).

It will be clear from Fig. 1a that the number of 19S AFC formed after the test injection of antigen fell sharply a week after the induction of tolerance and that appreciable recovery had occurred after 3 weeks. However, further recovery proceeded slowly, and even in the case of immunization after 13 weeks the number of 19S AFC in the spleen of the tolerant animals was significantly less (P 0.001) than in the control mice. After 6 months the immune response of the mice of all groups was virtually the same. Similar results were obtained when the number of 7S AFC in the spleen was tested (Fig. 1b): for a period of 2 months the immunoreactivity of the tolerant animals, determined by this test, was significantly lower than in the control. This series of experiments showed that the antibody-forming function not only of the spleen, but also of the lymph glands, was disturbed (Fig. 1c), i.e., the tolerance was not local but general in character.

TABLE 1. Cross Transplanation of Spleen Cells of Tolerant and Normal Mice

Index	Number of 19S AFC in spleen		
	experiment	control	P
Reaction of tolerant (experiment) donor mice and of those receiving CP only	5 333 (3 133–9 078) $n=21$	30 020 (19 820–46 030) $n=19$	<0,001
Reaction of spleen cells of tolerant mice (experiment) and of mice receiving CP alone (control) after transplantation into normal recipients	1 318 (861 – 2 018) $n=29$	4 074 (2 624 – 6 324) $n=24$	0,001
Reaction of spleen cells of normal mice after tranplantation into tolerant (experiment) recipients and into recipients receiving CP only (control)	5 420 (3 793–7 745) $n=23$	19 190 (14 490–25 410) $n=24$	<0,001

Note: Here and in Table 2 the geometric mean values and confidence limits for PP < 0.05 are given; n denotes number of animals.

TABLE 2. Effect of Serum of Tolerant Mice on Immune Response

Source of serum	Number of 19S AFC in spleen of recipients of serum	
	normal	absorbed by red cells
Mice 3 weeks after induction of tolerance	39 170 (24 550–62 370) $n=18$	115 900 (82 040–163 700) $n=11$
Mice 1 week after induction of tolerance	131 500 (87 500–197 700) $n=6$	145 900 (88 310–241 000) $n=7$
Normal mice or mice receiving CP	121 600 (86 900–169 800) $n=17$	109 100 (78 700–151 400) $n=8$
Serum not injected	137 700 (109 600–173 000) $n=22$	

The experiments thus showed that the tolerance arising in mice after injection of SRBC and CP and characterized by inhibition of the formation of 19S and 7S AFC in lymphoid tissue persists for at least 2-3 months.

Several workers have shown that the tolerance induced in animals by the combined injection of foreign red cells and CP can be prolonged by additional injections of antigen [4, 11, 14], in agreement with the concept of the role of persistance of the antigen in maintenance of the state of tolerance [9, 17]. It was therefore important to determine the duration of preservation of the antigen in the tolerant mice. To do this, (CBA × C57BL/6)F_1 mice received an intravenous injection of 100 million spleen cells of syngeneic mice, preliminarily sensitized with SRBC, at different times after the creation of tolerance. The number of 19S AFC in the recipients' spleen was counted 5 days later. The same method was used by Möller to determine the presence of antigen in immunized animals, but the sensitivity of the method in the modification now used was higher, for the cells injected were sensitized and not intact [16]. Sensitized cells are known to be capable of giving a secondary immune response to a small dose of antigen [1, 5] and even to antigen present in a nonimmunogenic form [2, 6].

Cells transplanted into tolerant mice 1 and 7 days after the induction of tolerance gave a well marked reaction commensurate with the reaction of the same cells to 1 million SRBC (Fig. 2; 1st control). Transplanation after 2 weeks gave a weaker reaction, but the level of AFC in the spleen of the experimental group of mice was significantly higher than in the control animals treated with CP alone, into which the cells were injected without antigen (2nd control). Finally, cells transplanted into tolerant mice 3 weeks after the induction of tolerance in the animals gave no immune reaction.

It can be concluded from these results that in mice tolerant to SRBC for a period of 2 weeks (or rather longer) after injection of SRBC and CP an antigen is present in the body, but by the end of the 3rd week its

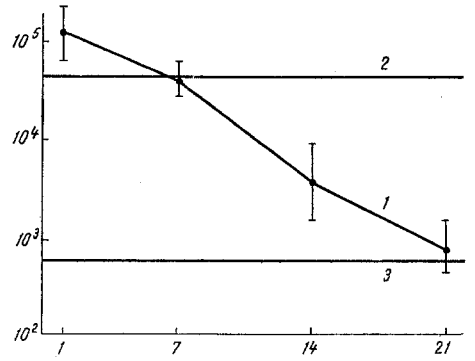

Fig. 2. Persistence of nitrogen in tolerant mice. Ordinate, number of 19S AFC in the spleen; abscissa, time of transplantation of cells after induction of tolerance (in days). 1) Reaction of sensitized cells transplanted into tolerant mice; 2) reaction of sensitized donor mice to injection of 1×10^6 SRBC (1st control); 3) reaction of sensitized cells injected into mice receiving CP alone (2nd control).

content has fallen to a level which could not be detected by the method used (less than 0.015% of the injected dose).

In the experiments of series 2 the reactivity of cells of the lymphoid tissue of tolerant animals was investigated in culture in vivo at a time when the antigen concentration in the body had fallen to below the threshold. Experiments were carried out on (CBA × C57BL/6)F_1 mice 3 or 4 weeks after induction of tolerance to SRBC in them. Spleen cells of tolerant animals (100 million) or of mice which received CP only (control) were transplanted together with 5×10^8 SRBC into intact syngeneic recipients which had first been irradiated in a dose of 700 R. On the 5th day the number of 19S AFC was determined in the recipients' spleen. The immune reaction to the same dose of antigen given by the spleen cells of intact mice transplanted into irradiated tolerant mice or into irradiated mice receiving only CP previously also was investigated.

As Table 1 shows, while the donor mice remained tolerant their spleen cells were characterized by lowered immunoreactivity in culture in vivo by comparison with the control. Meanwhile conditions inhibiting the immune reaction of normal cells transplanted into these mice had arisen in the tolerant mice. Incidentally, not all the experiments of this series gave unequivocal results. In one of them the reaction of the normal cells in the tolerant recipients was not suppressed, while in another the lymphoid cells of the tolerant mice in culture in vivo reacted to antigen in the same way as the control.

It can therefore be concluded from the results of these experiments that the tolerance of animals to SRBC 3-4 weeks after its induction by means of CP is due both to a deficiency of immunocompetent cells and to the influence of a certain agent which inhibits the immune response; often a combination of both factors is observed.

To ascertain the nature of this agent the effect of the serum of the tolerant mice on the immune response of normal animals was studied. Experiments were carried out on noninbred mice. Blood was taken from the donor mice 1 or 3 weeks after injection of 6.2 billion SRBC and of CP. The resulting serum was inactivated, sterilized by passing it through a Millipore filter, and injected in a dose of 0.5 ml intravenously into intact mice, which were given an intravenous injection of 5×10^8 SRBC 24 h later. The number of 19S AFC in the spleen was determined 4 days later. Serum of normal mice or of mice treated with CP only was injected into the control animals (Table 2).

Table 2 shows that serum obtained from the mice 3 weeks after tolerance had been induced in them inhibited the immune response ($P < 0.001$), whereas the serum of the control animals or that taken from the mice 1 week after the induction of tolerance had no such action. Treatment with SRBC caused the serum to lose its depressive properties. Hemolysins were absent from the serum of both tolerant and control mice (titer < 1:5) and hemagglutinins were present in low titers (< 1:80). If, however, hemaggultinins were determined in the presence of antiglobulin serum, their titer in the serum of the tolerant mice rose to 1:1280, whereas in the serum of the control animals it did not exceed 1:160. Absorption of the sera with SRBC lowered the hemagglutinin titer to <1:5.

The results show that the depressive acgion of the serum obtained from 3 weeks after induction of tolerance was associated with the hemagglutinating antibodies which it contained. These antibodies also were responsible, evidently, for inhibition of the immune response of the normal lymphoid cells transplanted into the tolerant mice, if the transplanation was carried out 3-4 weeks after the creation of tolerance in the recipient mice (Table 1).

The following conclusion can be drawn from the results described in this paper in conjunction with those obtained previously [3]. The tolerance arising in mice as a result of the combined administration of

SRBC and CP in its initial stage (up to 3 weeks) is "classical" in the sense that it is due entirely to a deficiency of immunocompetent cells. This is followed by stage II, when the decrease in reactivity of the population of lymphoid cells is no longer the only factor determining the state of tolerance: circulating antibodies possessing a depressive action appear during this period. The combination of both factors gives rise to the partial immunological areactivity which could be detected until 3 months. It is logical to conclude that in the last weeks of this period the depression of the immune response depends entirely on the presence of antibodies (stage III).

A problem which remains unsolved is that of the source and nature of the antibodies formed in the tolerant animals in the absence of additional antigenic stimulation (before the test injection of SRBC). Possibly not all the cells stimulated by the primary injection of antigen (6.2 billion SRBC) are sensitive to the action of CP. A few of these cells, which remain capable of differentiation and of forming antibodies, produce antibodies which in stage I of tolerance are masked by the excess of antigen in the body. After elimination of the greater part of the antigen these antibodies appear in the serum and exert an immunodepressive action. On the other hand it can be postulated that antibody formation in tolerant animals, is a reflection of the loss of tolerance after the concentration of antigen in the body has fallen below the critical level. The newly formed immunocompetent cells begin to produce antibodies in response to the action of the residual amount of antigen. These cells cannot be determined by the local hemolysis in gel test for the antibodies produced by them possess extremely low hemolytic activity, which is not demonstrable even in the presence of antiglobulin serum. These are possibly incomplete antibodies of the type revealed by the Coombs' test [7].

A comparison of these results with those described in the literature suggests that a sequence of change and the coexistence of different mechanisms regulating the immune response of tolerant animals are possible not only in the tolerance induced by administration of SRBC and CP, but also in its other forms (overloading, Dresser's phenomenon, tolerance to skin grafting, etc.). The formation of a special class of antibodies (in this case, AFC) in tolerant animals, performing a regulatory function, may therefore be a more general rule which must be taken into consideration in the analysis of the mechanisms of tolerance.

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