# EFFECT OF CYCLOPHOSPHAMIDE ON SYNTHESIS OF ANTIBODIES AND NONSPECIFIC IMMUNOGLOBULINS IN MOUSE SPLEEN CELLS

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Intraperitoneal injection of cyclophosphamide (CP) into mice in a dose of 200 mg/kg body weight leads after 2-3 weeks to a marked acceleration of the formation of nonspecific immunoglobulins (NIG) by spleen cells in vitro. In the early period (3-7 days) after injection of CP the acceleration of NIG production was due mainly to synthesis of macroglobulins; after 2 weeks the formation of both macroglobulins and IgG was increased. Normal NIG synthesis was not restored until at least 1 month later. Injection of antigen (sheep's red cells) against the background of hyperproduction of NIG led to a decrease in the latter. The formation of antibodies against red cells in mice treated with CP was at a lower level at all times of the investigation than in control immunized animals.

KEY WORDS: cyclophosphamide; antibodies; nonspecific immunoglobulins.

Cyclophosphamide (CP) is known as an inhibitor of antibody formation [3], depressing the proliferation [7, 9] and differentiation [8] of B lymphocytes.

The object of this investigation was to study the late effects of CP on the synthesis of antibodies and nonspecific immunoglobulins in adult animals.

## EXPERIMENTAL METHOD

Experiments were carried out on CBA mice and (CBA × C57BL/6)$F_1$ hybrids. CP was injected intraperitoneally in a dose of 200 mg/kg body weight. After various time intervals the spleen was removed from the control and experimental animals, its cells were incubated in Eagle's medium containing $^{14}$C-glycine, and the quantity of nonspecific immunoglobulins (NIG) synthesized was determined from the increase in radioactivity on an immunosorbent containing rabbit antibodies against mouse IgG [1].

To determine the effect of CP on antibody formation, intact mice and mice receiving CP were immunized with sheep's red cells ($5 \times 10^8$ cells intravenously). The spleen was removed on the 4th day and the cells incubated as described above.

Antibody formation against red cells was judged from the number of antibody-forming cells (AFC) in the spleen, determined by Jerne's method, and from the titer of hemagglutinins and hemolysins in the blood serum.

NIG synthesized by the cells of the immune spleen were determined after exhaustion of the culture medium with sheep's red cells, also by the aid of an immunosorbent [1].

To determine the classes of NIG synthesized under the different conditions the culture medium was fractionated at 4°C on a column with Sephadex G-200 (26 × 100 cm), equilibrated with 0.1 M Tris buffer, pH 7.6, containing 1 M NaCl. In some experiments, before the culture medium was applied to the column, it was

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treated with dithiothreitol and monoiodoacetamide [6] to produce dissociation of the macroglobulins in it. NIG in the eluted fractions was determined with the aid of an immunosorbent (the volume was 2.7 or 5.4 ml).

Radioactivity was counted with a BFL25 end-type counter and an Intertechnique SL-40 scintillation counter.

## EXPERIMENTAL RESULTS

Injection of CP into the mice led after 2-3 weeks to a sharp (by three to four times) increase in NIG synthesis. Their formation remained increased for at least 3 weeks after a single injection of CP into the animals, after which the NIG level fell gradually, to reach in 1 month the level observed in normal animals. Data on changes in NIG synthesis depending on the time elapsing after injection of CP into the mice are given in Table 1. Since the absolute values of incorporation of the label varied greatly from one experiment to another, the results are given in percentages of NIG synthesis in intact animals.

The next step was to discover which classes of immunoglobulins were responsible for the increase in NIG in the animals treated with CP. For this purpose, culture fluid obtained during incubation of spleen cells taken 3, 7, and 14 days after the injection of CP was fractionated on a column with Sephadex G-200. The results showed that after 3 and 7 days mainly macroglobulins were synthesized; after 14 days the formation of both macroglobulins and IgG was sharply increased (Fig. 1, curve 1). The main class of NIG synthesized in vitro in the intact mice was macroglobulins (Fig. 1, curve 2); in animals immunized with red cells synthesis of both macroglobulins and IgG was increased (Fig. 1, curve 3).

Macroglobulins synthesized by intact spleen cells had a lower molecular weight than those produced by cells of the immune spleen, and the latter, in turn, were "lighter" than macroglobulins synthesized by cells from mice receiving CP.

After discovering these differences in the distribution of the macrocomponents in the three different cases described above, it was decided to test whether the heavy components of the culture medium during investigation of mice receiving CP was in fact macroglobulin. For this purpose the medium was reduced and alkylated under mild conditions, leading to dissociation of the macroglobulins into subunits [6]. After the treatment the peak of the heavy material disappeared completely, showing it to be macroglobulin in nature. The same result was obtained by fractionation of the culture fluid containing NIG of immunized animals on a column with Sephadex G-200.

The "collapse" of NIG synthesis observed in this investigation under the influence of CP was to some degree unexpected. A previous study [4] showed that hyperregeneration, following 1 week after aplasia of the hematopoietic and lymphoid cells caused by injection of CP, affects mainly the erythroid and myeloid cells of the spleen; it is not until after 3 weeks that the lymphoid tissue returns to normal.

It is also known that the level of cells forming rosettes with sheep's red cells 1-2 weeks after injection of CP is either the same as the control or actually a little lower [2]. Finally, in many investigations immunocompetence was found to be depressed as a result of the injection of CP, and this was confirmed by the results of the present investigation (see below). The mechanism of formation of the large quantities of NIG under the influence of CP thus still remains unexplained.

The relatively small increase in the number of "background" cells producing antibodies against sheep's red cells at a time when macroglobulin synthesis was stimulated by CP (Table 2) and the absence of any increase in the titers of hemagglutinins and hemolysins, which did not exceed 1:10 in the blood sera of the experimental animals, i.e., which were also within normal limits, deserve attention.

TABLE 1. Effect of CP on NIG Synthesis Depending on Time of Its Injection

| Number of mice in group | Time after injection of CP (days) | NIG synthesis (% of control) |
|---|---|---|
| 43 | — | 100,0±6,6 |
| 13 | 14—18 | 320,8±59,6 |
| 9 | 21—25 | 178,8±38,9 |
| 30 | 28—30 | 110,9±8,8 |

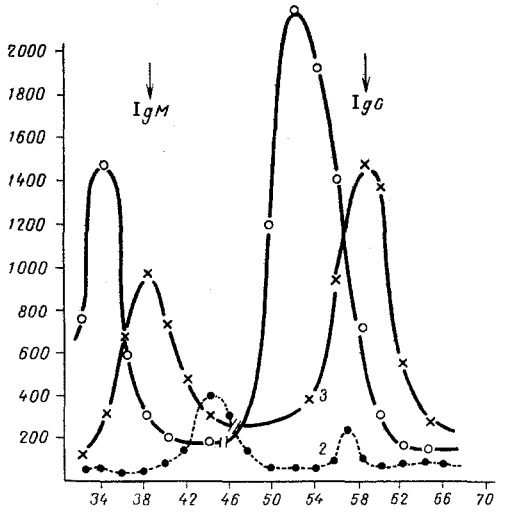


Fig. 1. Distribution of immunoglobulins synthesized by spleen cells from intact mice, mice immunized with red cells, and mice treated with cyclophosphamide, during gel filtration on Sephadex G-200 column: 1) NIG synthesized by spleen cells 14 days after injection of CP into mice; 2) NIG synthesized by spleen cells of intact animals; 3) NIG synthesized by spleen cells of mice immunized with sheep's red cells. Abscissa, fraction No.; ordinate, $^{14}C$ activity (counts/min).

TABLE 2. Synthesis of Antibodies and NIG by Spleen Cells of Mice Immunized with Red Cells Depending on Time after Injection of CP

| CP injected | Time after injection of CP (days) | Number of AFC (per $10^6$ cells) | | Quantity of NIG (counts/min/$10^8$ cells) | |
|---|---|---|---|---|---|
| | | before test | after test | before test | after test |
| — | — | 0,13±0,04 (12) | 281±73 (4) | 648±196 (5) | 1215±182 (4) |
| + | 14—18 | 0,20±0,05 (13) | 86±33 (6) | 2628±400 (5) | 1099±163 (6) |
| — | — | —* | 258±20 (3) | 319±43 (9) | 492±63 (3) |
| + | 21—25 | —* | 32±13 (6) | 628±118 (7) | 376±62 (6) |
| — | — | —* | 540±76 (5) | 185±60 (5) | 499±58 (5) |
| + | 28—30 | —* | 232±42 (4) | 127±25 (5) | 381±68 (4) |

*Not determined. Number of animals in group shown in parentheses.

The absence of increase in the number of cells producing antibodies against red cells indicates that the NIG synthesized under the influence of CP differ from the polyclonal NIG formed through the action of nonspecific mitogens [5].

It was shown previously that treatment of mice with CP does not prevent the appearance of large numbers of AFC when the animals are immunized 14 days later with sheep's red cells [1]. Similar results were obtained in the present investigation. There was a sharp increase in the number of AFC in the spleen of the immunized animals treated previously with CP, and the titers of hemagglutinin and hemolysis in their blood sera rose to 1:80-1:640. However, at all times of the action of CP investigated, the number of AFC in the spleen after a test injection of antigen was lower than in mice not receiving CP (Table 2).

The sharp decrease in NIG synthesis when antigen was injected into the animals in the early stage of the action of CP was unexpected. For instance, whereas in the intact mice immunization with red cells stimulated NIG synthesis by about 1.5-2.5 times, in animals receiving CP 2-3 weeks beforehand NIG synthesis, by contrast, was reduced by 33-50%. Ability to react to antigen by increased synthesis of NIG was not restored until at least a month later, and moreover, not in all experiments.

Since the antigenic stimulus increasing NIG synthesis in the control animals did the opposite in mice receiving CP shortly beforehand, and depressed it, ultimately the initial differences in the intensity of formation of NIG in the two groups of animals were equalized. Under the influence of the antigenic stimulus, individual fluctuations in the intensity of NIG synthesis also were reduced: As special calculations showed, the standard deviations, expressed as percentages of the mean, ranged from 34 to 75 in the different groups before injection of the antigen, but only from 22 to 40 after its injection (a decrease in five of the six experiments).

The mechanism of the normalizing action of the antigen is not yet clear. The possibility cannot be ruled out that the stimulating and inhibitory effect of the antigen on NIG synthesis operates in general at different levels.

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