

POTENTIATION OF T-CELL-MEDIATED IMMUNITY BY
SELECTIVE SUPPRESSION OF ANTIBODY FORMATION
WITH CYCLOPHOSPHAMIDE*

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Mice develop delayed-type hypersensitivity (DTH)¹ to heterologous red cells provided that dose and route of immunization are appropriately chosen (1). No adjuvant is needed, but the hypersensitivity will be transient and of low intensity if the dose of antigen is too large. For example, the dose of sheep red blood cells (SRBC) which gives maximum antibody production by intravenous immunization causes only a fleeting and barely discernible episode of hypersensitivity. The suppression of DTH is due to blocking factors which are formed by the interaction of antigen and antibody (2). The mediators of DTH can be freed from the influence of this normal inhibitory mechanism by splenectomy (2), by infection with BCG (3), or by treatment with drugs such as cyclophosphamide (CY) which act differentially on B lymphocytes (4-6). Since it was known that DTH reactions tend to be more prolonged in CY-treated animals (7, 8), this drug has been used in the present experiments to investigate the role of antibody in the regulation of T-cell activity.

Materials and Methods

Methods have been described elsewhere for measuring hemagglutinin titers and DTH (2), and lymphoproliferative responses and numbers of plaque-forming cells (PFC) in popliteal lymph nodes (9, 10). The transfer of DTH with dissociated spleen cells has also been described (11).

Animals.—Specific pathogen-free male and female mice of the CD-1 strain (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were used at 5 or 6 wk of age. C57BL mice, also from a specific pathogen-free colony, were used in one experiment.

Antigens.—SRBC were obtained from the same animal twice weekly. Cells were collected and stored in Alsever's solution at 4°C. They were washed three times with normal saline and suspended to known density by hemacytometer count.

CY.—Cytosoxan was donated by Mead Johnson & Co., Evansville, Ind. It was dissolved in

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¹ *Abbreviations used in this paper:* CY, cyclophosphamide; DTH, delayed-type hypersensitivity; HAG, hemagglutinating antibody; PFC, plaque-forming cells; SRBC, sheep red blood cells.

sterile saline (20 mg/ml) and was administered intravenously as a single dose of 200 mg/kg body weight.

RESULTS

Effect of CY on the Response to Varying Doses of SRBC.—When varying doses of SRBC were given intravenously to normal and CY-treated mice, the latter developed high levels of DTH in response to doses of SRBC which caused partial inhibition and early decay of DTH in untreated mice (Fig. 1). The C57BL mice differed from CD-1 mice used in all other experiments in being

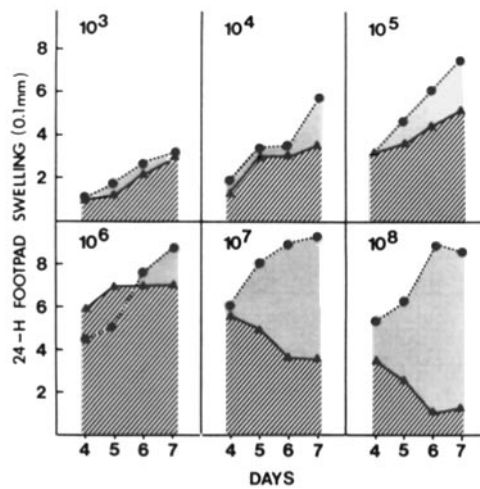


FIG. 1. Levels of DTH measured on days 4–7 in normal (▲—▲) and CY-treated (●—●) C57BL mice which had been sensitized intravenously with the indicated doses of SRBC. CY (200 mg/kg) was given 2 days before immunization. The area of stippling represents the augmentation of DTH attributable to the immunosuppressant action of CY. Means of five mice.

less responsive to SRBC (1, 2). It was nonetheless apparent that DTH was potentiated because CY suppresses the inhibitory mechanism that is activated by a large dose of antigen.

The Effect of Varying the Interval between CY and Antigen Injections.—DTH reaches its peak and begins to decay on day 4 in mice immunized in one footpad with 10^8 SRBC, the optimum dose for sensitization by this route (1). The effect of CY on DTH was therefore measured at two time points, days 4 and 8. The drug's immediate influence on the cellular response that leads to the formation of activated T cells could thus be compared with its influence on the normal decay of DTH.

The average levels of DTH measured on days 4 and 8 in untreated mice immunized with 10^8 SRBC in one hind footpad are indicated by the interrupted horizontal lines of Fig. 2. The levels of DTH observed in animals treated with

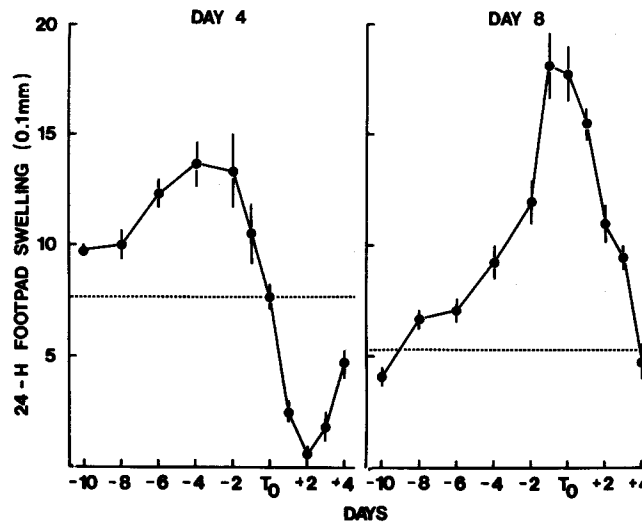


FIG. 2. Levels of DTH measured on days 4 and 8 in mice sensitized on day T_0 by footpad inoculation of 10^8 SRBC. On the days indicated, mice of separate groups were injected intravenously with CY in a dose equivalent to 200 mg/kg. The interrupted horizontal lines represent the mean levels of DTH found on days 4 and 8 in sensitized but untreated mice. Means of $5 \pm$ SEM.

CY at varying times relative to the same sensitizing dose of SRBC are represented by the continuous lines. The effect of CY on the early, or inductive, phase of the immune response was biphasic: DTH was potentiated in mice given CY before antigenic stimulation and depressed by treatment given after immunization. However, the episode of depressed T-cell activity had disappeared by day 8, and been replaced by an impressive degree of enhancement except in animals receiving CY on day +4.

A mean footpad swelling of more than 18 U (1.8 mm), as recorded on day 8 in Fig. 2, represents a very gross reaction indeed. Moreover, and in confirmation of the observations of Turk et al. (7), the reactions elicited in the footpads of CY-treated mice persisted for at least 72 h. One such reaction is illustrated in the accompanying paper (3).

Adoptive Transfer of DTH with Spleen Cells from CY-Treated Donors to Normal and CY-Treated Recipients.—The immediate and very marked depression of DTH in animals treated with CY on day +2 suggested that T cells may not be entirely unaffected by this drug. But as circulating monocytes are required for the expression of DTH (12), and as CY interferes with the formation of this cell type (13), more evidence was needed. A composite experiment was therefore performed in order to determine whether the phase of depressed reactivity in Fig. 2 was due to destruction of specific mediators or to arrested production of monocytes.

Donor mice were immunized intravenously with the optimal sensitizing dose

of 10^5 SRBC. Animals of one donor group (A) were left untreated, and those of donor groups B and C were given CY (200 mg/kg) 2 days before or after immunization, respectively. The level of hypersensitivity was measured at the time of cell transfer (day 4) in a sampling of five mice from each panel of donors. Spleen cells from each panel were pooled, and each recipient was given one spleen equivalent intravenously (approximately 10^8 cells) 1 h before testing for DTH. Cells from donor panel B were also used for transfer to CY-treated recipients which had been treated with CY (200 mg/kg) at intervals ranging from 0 to 5 days before cell transfer.

As in a previous study (2), the transfer of one spleen equivalent of cells from untreated hypersensitive donors (group A) rendered recipients about half as sensitive as were the donors themselves (Fig. 3). Cells from donors treated with CY 2 days before immunization (group B) were much more active in transferring hypersensitivity, though the donors themselves were distinctly less hypersensitive at this time. Donors given CY 2 days after immunization (group C) were virtually anergic at the time of cell transfer and their spleen cells failed to confer a significant level of DTH on recipients in whom monocyte production was presumably normal ($P < 0.2$). CY had obviously damaged the mediators of DTH in donors of this group. This effect of CY on the specific mediators of DTH was convincingly dissociated from effects caused by damage to monocyte precursors. The observations recorded on the right in Fig. 3 show that DTH reactions could not be elicited at all in animals given CY 4 days before cell transfer. Yet CY remains active in recipients for only about 48 h (see Fig. 6). It follows that the specific mediators of DTH would have been

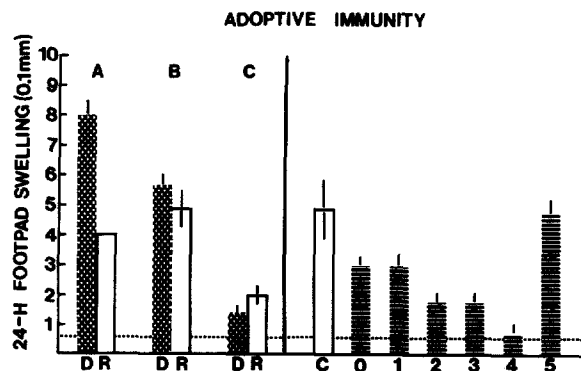


FIG. 3. Left: Levels of DTH found in the donors (D) and the adoptively sensitized recipients (R) of spleen cells (one spleen equivalent, 1×10^8) from mice sensitized intravenously with 10^5 SRBC 4 days before cell transfer. The donors of group A were untreated, and those of group B and C received CY (200 mg/kg) 2 days before and 2 days after sensitization, respectively. Means of $5 \pm$ SEM. Right: Levels of DTH measured in adoptively sensitized mice given 10^8 spleen cells from donor panel B. The cells were transferred to untreated recipients (C) or to recipients on days 0-5 after treatment with CY (200 mg/kg). Means of $5 \pm$ SEM.

damaged by CY in this group of adoptively sensitized recipients. Only the absence of another element, presumably the monocyte (12), could explain their failure to react. The timing was consistent with this view, for McGregor and Koster (13) have shown in rats that the supply of monocytes for mobilization into a peritoneal exudate becomes exhausted 4 days after a large single dose of CY. But as circulating monocytes do not disappear immediately after treatment, the depressed reactions observed in recipients given CY at the time of transfer, or 1 day later, were probably due to drug damage sustained by specific mediators after transfer.

Development and Persistence of DTH in CY-Treated Mice.—The action of CY on the T-cell component of the immune response is clearly most marked in the period immediately surrounding the time of sensitization. A more detailed picture of its influence at this time was obtained by following the development and persistence of DTH in groups of mice which were given CY before (T_{-2}), with (T_0), or after (T_{+2}) sensitization by a footpad injection of 10^8 SRBC. Sensitized, but untreated, controls were tested at each time point.

Fig. 4 shows that CY caused a marked increase in the level and longevity of DTH whether given before, with, or after antigen, but the peak was reached at progressively later times. This indicates that the T-cell response is under severe constraint for a well defined period after administration of CY. The antigenic stimulus from a footpad inoculation must therefore persist for long enough to allow resumption of T-cell induction after the effect of CY has worn off. Even when the same dose of SRBC (10^8) was given intravenously, a rebound from the suppressive effects of CY occurred, but the ultimate level of DTH was much lower (Fig. 5). This presumably means that a larger fraction

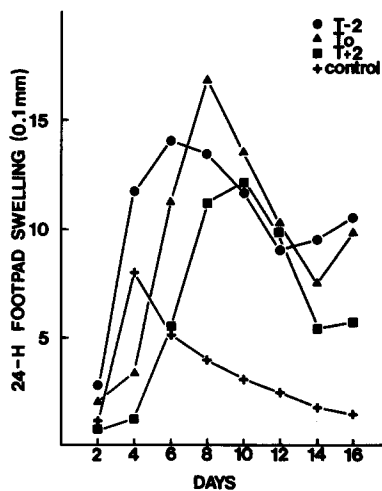


FIG. 4. Levels of DTH measured in control and CY-treated mice at intervals after sensitization by footpad inoculation of 10^8 SRBC. The times of injecting CY relative to antigen inoculation were as indicated. Means of five mice.

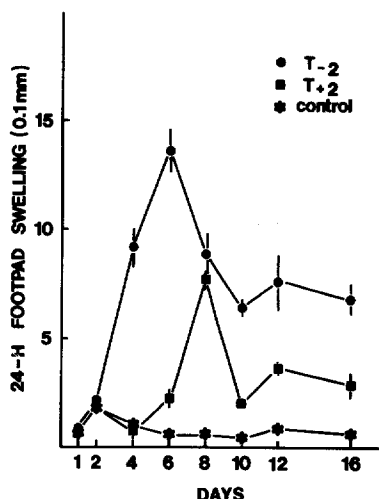


FIG. 5. Levels of DTH measured in control and CY-treated mice at intervals after sensitization by an intravenous injection of 10^8 SRBC. The times of injecting CY relative to antigen inoculation were as indicated. Means of $5 \pm$ SEM.

of the immunocompetent pool of precursor T cells is recruited by intravenous immunization and is destroyed by CY given on day +2.

Effect of CY on the Lymph Node Response to SRBC.—The rates of cell proliferation (^3H)thymidine incorporation into DNA) and numbers of PFC were measured on successive days in the popliteal lymph nodes of mice immunized in one hind footpad with 10^8 SRBC. The node response of untreated controls was compared with that of mice given CY before (T_{-2}), with (T_0), or after (T_{+2}) the sensitizing inoculum of SRBC. The corresponding levels of DTH and serum levels of hemagglutinating antibody (HAG) were also measured.

Fig. 6 A shows that the proliferative response in the popliteal lymph node was arrested for about 48 h by CY given at the time of antigen inoculation (T_0). Only in this group was it possible to see how long the drug remains active because the effective concentration of drug had already fallen before the onset of cell proliferation in mice given CY before antigen (T_{-2}), and the effect was so severe when CY was given after antigen (T_{+2}) that cell proliferation never resumed a normal pace.

PFC production and cell proliferation rates were closely correlated. When the lymphoproliferative response was delayed by CY, the time taken to reach maximum numbers of PFC in the regional node was correspondingly increased (Fig. 6 B), and HAG's took longer to appear in the blood (Fig. 6 C).

The development of DTH was followed for only 8 days (Fig. 6 D), but the trends were identical with those recorded in Fig. 4. It seems, therefore, that peak levels of hypersensitivity were reached on days 4, 6, 8, and 10 in untreated mice and those given CY on days -2, 0, and +2, respectively.

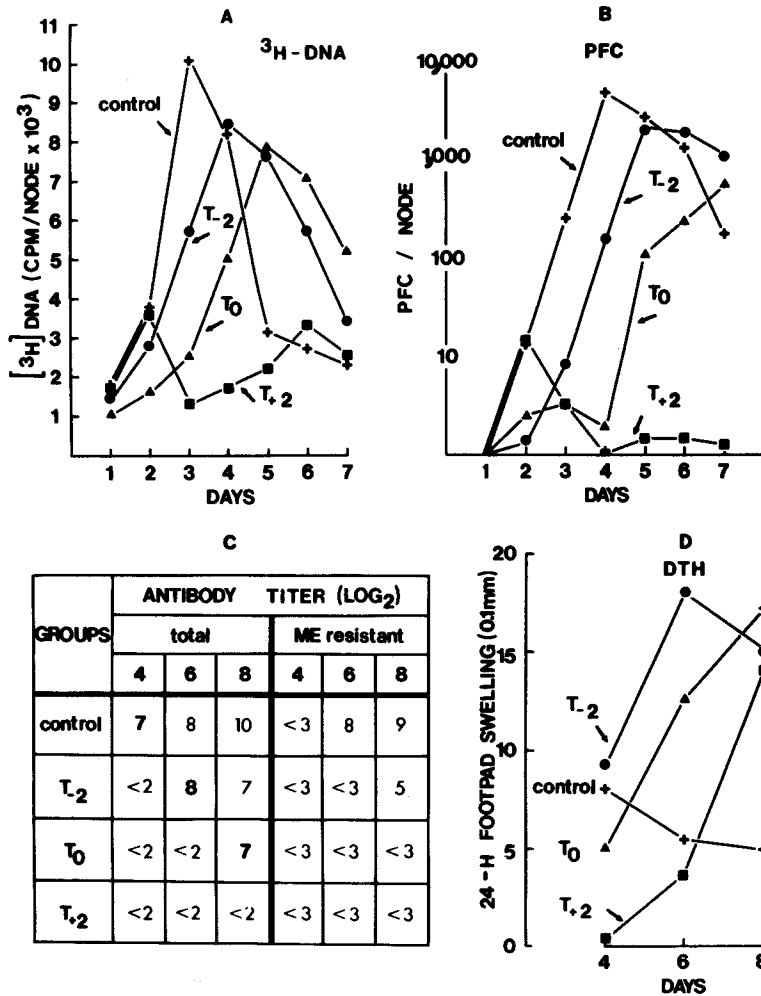


FIG. 6. Levels of thymidine incorporation (A) and numbers of direct PFC (B) in poplitea lymph nodes of mice treated with CY at various times relative to footpad inoculation with 10⁸ SRBC. The corresponding antibody titers and levels of DTH elicited in the opposite foot are recorded in C and D. Means of five mice.

DISCUSSION

Humoral and cellular immunity tend to be mutually exclusive (14). Witness, for example, the contrasting responses of normal and splenectomized mice to a massive intravenous injection of SRBC: The former produce antibody but no DTH, while the latter develop DTH but make little or no antibody (1). This demonstrates, among other things, that antigen alone cannot interfere with the function or formation of activated T cells. By way of confirmation, CY-treated mice developed high levels of DTH with doses of SRBC that blocked T-cell activity completely in untreated mice (Fig. 1). In fact, CY enhanced DTH only to the extent that it released the T-cell

response from the feedback inhibition that normally accompanies the antibody response (2).

The fact that DTH reactions are longer lasting in CY-treated animals has been interpreted as evidence of a differential effect on T and B cells (5-7), a view that is supported by the diminished B-cell populations found in the blood and lymphoid tissues after treatment (4-6). It is certain, however, that CY does not act exclusively on B cells. For one thing, it prolongs the survival of skin allografts (15). And as the present findings show, it destroys the mediators of DTH if administered at the right time. Indeed, as others have stressed (16), timing critically affects the immunosuppression achieved with CY. In the present experiments three different effects were obtained by varying the time of treatment in relation to the antigenic stimulus. A single dose of CY which increased the level of T-cell activity (DTH) when given as much as 10 days before immunization caused a profound, if transient, depression when given 2 days after immunization (Fig. 2). Still later in the immune response, when DTH was already established, CY had little apparent effect on T-cell activity apart from its influence on monocyte production (Fig. 3).

The reason for these effects of timing rests with the mode of action of CY and the physiological state of participating lymphocyte populations before and after becoming engaged by an antigenic stimulus. The proliferative response of the popliteal lymph node was delayed for 48 h in mice immunized and treated simultaneously with CY (T_0 , Fig. 6 A). This places an upper limit of 2 days on the duration of the cytostatic action of CY in the mouse. Yet animals treated 4, 6, or even 8 days before immunization developed abnormally high levels of DTH by day 4 (Fig. 2). CY obviously has a long-lasting effect on the cells responsible for the inhibition of T cells. Moreover, the affected cells clearly differ from those which mediate DTH in being susceptible at all times to a drug that is active only at certain stages of the mitotic cycle (17). They must derive, therefore, from rapidly replicating precursors. A relevant, and once disputed, finding of Nossal and Mäkelä (18) showed that a very high (if not improbable) proportion of antibody-secreting cells became labeled by an injection of tritiated thymidine given 2 h before antigen. Though the labeling rates were probably inflated by reutilization, this finding gains significance from the fact that bone marrow lymphocytes (predominantly Ig-bearing B cells) have a very high turnover rate (19) and are a rich source of antibody-forming precursors. Moreover, Strober has shown that the B cells which become engaged by a primary antigenic stimulus are nonrecirculating cells which are destroyed by a mitotic poison (20).

While an origin in rapidly replicating precursors explains why B cells should be so vulnerable to the toxic action of CY, and why the T-cell-potentiating effect of the drug should be accompanied by a delay in the appearance of PFC in regional lymph nodes (Fig. 6 B) and of hemagglutinins in the serum (Fig. 6 C), there is no doubt that CY exerts a more depressive effect on antibody production if given after antigenic stimulation (16). This may be due to interference with helper cell activity. If helper cells and the mediators of DTH

belong to the same cell population (21), they would certainly be damaged by CY given 1-3 days after antigen. Although resting T cells are apparently unaffected by CY, those which mediate DTH are clearly vulnerable once they have been immunologically induced. The extreme susceptibility of dividing cells to the toxicity of CY was revealed by the abrupt cessation of thymidine incorporation by responding nodes when CY was given on day +2 (Fig. 6). As a result, PFC failed to appear in the regional node, antibody could not be detected in serum, and DTH was absent on day 4 when it should have been reaching its peak. This last effect was due mainly to destruction of specific mediator cells, as transfer studies showed (Fig. 3). It is important to note, however, that the T-cell response recovered from the severe depression caused by CY given when cells had begun to divide. Though cells did not resume proliferation on a large scale (Fig. 6 A), the output of activated T cells was enough to support a high level of DTH by day 8 (Fig. 6 D). This must mean that the antigenic stimulus from a footpad inoculation persists for long enough to recruit a new batch of precursors that were not part of the first wave of responding T cells, or that some cells recover from the effect of CY and go on to proliferate. In either case, the mediators of DTH obviously must come from this new crop of dividing cells. But many potential members of the population are undoubtedly annihilated by CY given on day +2, thus explaining why the ultimate levels of DTH are lower, especially in intravenously immunized animals (Fig. 5). The fact that even in these animals DTH reaches a higher level than in untreated controls speaks much for the constraint that is normally imposed on the T-cell response by feedback inhibition. Indeed, in attempting to raise the general level of T-cell activity by selective immunosuppression one is clearly limited by the circumstance that when timing is such as to achieve maximum suppression of antibody formation (and hence of feedback inhibition) the drug's effect encroaches upon the phase of T-cell proliferation and thus restricts the output of activated T cells. It would appear from the data of Fig. 4 that maximum levels of DTH are achieved when CY and a subcutaneous injection of antigen are given synchronously. Even though this regimen did not prevent antibody formation, it did give T cells the longest period of freedom to proliferate before antibody-forming cells appeared in the regional node where most of the activated T cells are presumably made.

SUMMARY

Delayed-type hypersensitivity (DTH) appears in mice immunized with less than an optimal immunogenic dose of sheep red blood cells (SRBC), but is blocked progressively as antibody production increases in response to larger doses of SRBC. Treatment with cyclophosphamide (CY) was shown to release T cells from this inhibitory influence of the humoral response, and cause enhancement of DTH. The magnitude of this enhancing effect on T-cell activity was markedly dependent on the time of treatment relative to the time of

immunization, and on the time chosen for measuring DTH. The reasons for these pronounced effects of timing are threefold: (a) CY given before antigenic stimulation has a long-lasting effect on antibody formation, but no apparent effect on the precursors of activated T cells. (b) After antigenic stimulation, T cells also become susceptible to CY. (c) The production of a nonspecific participant (monocyte) in the DTH reaction is also suppressed by CY, though the supply of circulating monocytes is not immediately affected by the drug.

The differential effect of CY on T and B lymphocytes depends on the differing physiological states of the majority of cells that make up these two populations. The former are resting cells that are insensitive to CY until exposed to specific antigen, while the latter are drawn from a rapidly replicating precursor pool and are susceptible to CY at all times.

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