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Cyclophosphamide intensifies the acquisition of allergic contact dermatitis in mice rendered B-cell deficient by heterologous anti-IgM antisera

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Summary. Treatment with cyclophosphamide (Cy) before contact sensitization regularly intensifies the induced sensitivity. The immunopotentiation is specific and appears due to toxicity for suppressor cells. It has been proposed that the target of Cy immunopotentiation is a suppressor B cell. We have studied allergic contact dermatitis (ACD) in mice rendered B-cell deficient by chronic treatment, beginning at birth, with a goat antiserum against mouse IgM. The ACD induced in these B-cell deficient mice was equal to that induced in intact mice. The hypersensitivity was readily and equally immunopotentiated by Cy in normal and in B-cell deficient mice. It appears that the suppressor cell that is the target of Cy immunopotentiation is not a B cell but rather a T cell.

INTRODUCTION

Pretreatment with cyclosphosphamide (Cy) potentiates allergic contact dermatitis (ACD) in the guinea-pig (Maguire & Ettore, 1967; Hunziger, 1968; Turk, Parker & Poulter, 1972). It is known that Cy preferentially depletes the B-cell areas of lymph nodes and spleen in guinea pigs and mice (Turk & Poulter, 1972). Since immunopotentiation by Cy could be reversed by passive transfer of viable spleen cells from guinea-pigs sensitized without Cy, (Katz, Parker, Sommer & Turk, 1974b), it appeared that immunopotentiation occurred because Cy was toxic for suppressor cells that were probably B cells. Indeed, experiments with fractionated spleen cells demonstrated that the pertinent suppressor cells had the surface characteristics of B cells (Katz, Parker & Turk, 1974a). Pretreatment with Cy also potentiates ACD in mice (Zembala & Asherson, 1976; Zembala, Asherson, Noworolski & Mayhew, 1976); the relevant suppressor cells have B-cell characteristics and it appears that during Cy immunopotentiation, the precursors of these suppressor B cells are selectively reduced.

Mice can be rendered profoundly B-cell depleted by long-term treatment, begun shortly after birth, with heterologous antisera directed against mouse μ-chain (Manning, 1975). Although the antiserum reacts only with B cells bearing IgM determinants on their surface, a reduction also results in B cells having IgG or IgA surface receptors, and perhaps of B cells with surface IgE (Dwyer, Rosenbaum & Lewis, 1976; Manning, Manning & Reed, 1976). It has been postulated that the deficiency extends to B cells of these other classes because such cells derive from B cells originally having surface receptors with IgM determinants. There is no comparable B-cell deficient model in the guinea-pig.

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We have studied ACD in mice rendered B-cell deficient by chronic treatment with a goat antiserum directed against mouse IgM. We have demonstrated that such mice can be sensitized to simple contact allergens as easily as normal mice and that this sensitivity can be immunopotentiated by Cy.

MATERIALS AND METHODS

Mice

We obtained breeding pairs of albino random-bred ICR/Ha mice from the Institute for Cancer Research (Fox Chase, Pa). Within 24 h of birth, all the mice of a given litter were injected i.p. with goat anti-mouse IgM serum *(vida infra)* and the injections continued on a thrice weekly schedule until termination of the experiment. Volumes of 0·05 ml were injected each time for the first 2 weeks and 0·1 ml later. Control mice were drawn from non-injected litters of the same age.

Immunosuppressive serum

Mouse myeloma protein, MOPC-104E (μ, λ), was purchased from Litton Bionetics, Rockville, MD. A goat was immunized by a series of biweekly subcutaneous injections of 0·5 mg MOPC-104E emulsified in Freund's complete adjuvant. Bleedings were made 10 days after each immunization. We used serum from a pool of bleedings that were taken between 1 and 2 years after the start of immunization. The goat antiserum was absorbed with ICR/Ha erythrocytes. The absorbed goat serum showed strong reactivity, by immunoprecipitation in gel, with the homologous myeloma protein MOPC-104E and gave reactions of identity with monospecific anti-mouse IgM purchased from two different sources.

Allergens

Oxazolone (4-ethoxy methylene-2-phenyl-oxazolone; B.D.H. Chemicals, Ltd, Poole, England) and NDMA (*p*-nitroso-N,N-dimethyl-aniline (K and K Laboratories, Plainview, N.J.) were prepared as fresh solutions immediately before use. Commercially obtained sheep erythrocytes (SRBC) were washed three times and diluted to desired final concentrations in phosphate buffered saline (PBS).

Sensitization and challenge

Mice were sensitized by a single application of allergen in acetone to a clipped site on the rear flank. They were tested for sensitivity by applying to the outer aspect of an ear 0·01 ml of 0·1% allergen in acetone. Baseline and subsequent measurements of thickness of the test site were made with an engineer's micrometer. (Asherson & Ptak, 1969).

Antibody determination

Agglutinating antibody to SRBC was measured in microtitre wells using two-fold serial dilutions of a 1:10 concentration of mouse sera in PBS.

Drug

Cyclophosphamide was obtained from the Meade Johnson Co., Evansville, IN.

Statistics

Statistical differences were analysed by the Mann-Whitney test.

RESULTS

Seven to eight week old, female ICR/Ha mice, that had been immunosuppressed from birth with antiserum directed against mouse IgM, were injected with Cy (150 mg/kg i.p.). Two days later they were contact sensitized with 2% oxazolone. Control groups that were sensitized in parallel included (1) ICR/Ha mice immunosuppressed with antiserum to mouse IgM but not given Cy and (2) normal ICR/Ha mice not pretreated with Cy. The protocol and results of this experiment are outlined in Table 1. The anti-IgM suppressed mice that were pretreated with Cy developed the most intense contact challenge reactions; there was no difference in the challenge reactions between the B-cell suppressed mice and the normal mice (neither group given Cy).

After completion of the readings of the ACD reactions, bleedings were taken from all test mice. The mice receiving the goat anti-serum had had their last injection 48 h previously. The sera from these bleedings were analysed by immunoprecipitation in gel for the presence of mouse IgM and for the presence of circulating goat antibody to mouse IgM. In every case, the sera of the mice chronically treated with the goat antiserum failed to contain detectable mouse IgM. In addition, these mice did have circulating goat anti-IgM as demonstrated by a reaction of their sera with mouse IgM; this reaction showed a line of identity with the goat antiserum with which they had been injected.

In a further experiment, ICR/Ha mice were sensitized to NDMA. Groups I and II were suppressed with

Table 1. Immunopotentiation of ACD to oxazolone with Cy in the B-cell deficient mouse

Group			Treatments*				
			Day −2	Day 0	Day 7	Day 8	Day 9
(7)	(anti-IgM)	I	Cy	Oxazolone	Oxazolone	23·1 ± 4·9	15·5 ± 3·1
(6)	(anti-IgM)	II	—	Sensitization	Challenge	13·4 ± 4·9	10·0 ± 3·6
(8)	—	III	—	Sensitization	Challenge	15·4 ± 5·5	10·8 ± 4·2
(8)	—	IV	—	—	Challenge	1·2 ± 0·6	−0·4 ± 1·2

* Three groups of mice were sensitized on the flank with 2% oxazolone. Groups I and II were chronically immunosuppressed with goat anti-IgM; group I received Cy 2 days before sensitization. On day 7 all three groups, as well as a toxicity control group, were challenged on the ear with 0·1% oxazolone. The average increase in ear thickness (mm × 10^{-2}) and standard deviation, 1 and 2 days later is shown. At 24 h, groups I > II, I > III ($P < 0.01$); at 48 h, groups I > II, I > III ($P < 0.05$).

goat anti-mouse IgM; groups I and III were pretreated with Cy (150 mg/kg i.p.) 2 days prior to sensitization. The protocol and results of this experiment are shown in Table 2. The challenge reactions in both groups (normal and anti-IgM suppressed) of Cy pretreated mice were significantly larger at 48 h than in the two groups not treated with Cy; there was no significant difference between the reactions at 24 h. In our experience, the 48 h challenge reactions of animals immunopotentiated with Cy are generally larger than the 24 h reactions; without Cy, the 48 h reactions are almost always smaller. Thus, differences between normal and immunopotentiated groups are sometimes demonstrated at 48 h that are only suggested at 24 h.

We have repeated this experiment in other normal and anti-IgM suppressed mice using 2% oxazolone as sensitizer and groups of ten with comparable results (data not shown). In the NDMA experiment, we compared the immune response to SRBC of the normal and of the anti-IgM treated mice. Immediately after the 48 h reading of the NDMA skin reactions, we injected i.p. all of the mice in groups I, II, III and IV with 0·1 ml of a 10% suspension of SRBC (Table 3). Seven days later they were reinjected in the same way. On day 14, all of the mice were bled and their sera tested for agglutinins against SRBC. The results are outlined in Table 3. No antibody directed against SRBC was found in any of the anti-IgM suppressed

Table 2. Enhanced 48 h. ACD reactions to NDMA in mice immunopotentiated with Cy

Group		Day 0	Day 0	Day 7	Day 8	Day 9
anti-IgM	I	Cy	NDMA	NDMA	14·6 ± 1·9	18·8 ± 6·4
anti-IgM	II	—	Sensitization	Challenge	12·6 ± 3·3	10·0 ± 3·5
—	III	Cy	Sensitization	Challenge	13·5 ± 4·7	16·6 ± 5·1
—	IV	—	Sensitization	Challenge	10·7 ± 3·1	7·8 ± 2·7
—	V	—	—	Challenge	2·6 ± 1·4	1·9 ± 0·9

Four groups of mice were sensitized on the flank with 10% NDMA. Groups I and II were chronically immunosuppressed with goat anti-IgM; groups I and III received Cy 2 days before sensitization. On day 7 all mice, as well as a toxicity control group, were challenged on the ear. The average increase in ear thickness (mm × 10^{-2}), and standard deviation, at 1 and 2 days following is shown. At 24 h, there was no significant difference between experimental groups; at 48 h groups I > II, I > IV, III > II and III > IV. ($P < 0.05$).

Table 3. Suppression of antibody formation to SRBC in B-cell deficient mice

Group		Treatment* Day 9	Day 16	Antibody titre† i	ii
(anti-IgM, Cy)	I	SRBC	SRBC	all < 1	all < 1
(anti-IgM)	II	SRBC	SRBC	all < 1	all < 1
(cy)	III	SRBC	SRBC	6,7,6,6,5	5,5,3,4,3
—	IV	SRBC	SRBC	6,4,6,3,6	4,3,3,2,3
—	V	—	—	all < 1	all < 1

* All of the mice from the NDMA experiment (see Table 2) were immunized twice, at weekly intervals, to SRBC beginning 9 days after contact sensitization. One week after the second immunization, they were bled and their sera tested for haemagglutinating activity against SRBC using an initial dilution of mouse sera of 1 : 10 in PBS.

† The titres are presented (i) before and (ii) after reduction with 2-mercaptoethanol as the $-\log 2$ of the highest serum dilution showing agglutination.

mice. Pretreatment of the normal mice with Cy 12 days prior to immunization failed to depress significantly antibody against SRBC (compare titres of groups III and IV).

In another experiment, we compared the relative immunopotentiation produced by different doses of Cy in anti-IgM suppressed mice. Three days prior to sensitization, one group of mice received 150 mg/kg of Cy, and a second was given 50 mg/kg of Cy. The allergenic stimulus was reduced by half in this experiment so as to decrease the sensitization of the control mice and thereby make more evident immunopotentiation by Cy. The three groups of sensitized mice, as well as a toxicity control group, were challenged 7 days later. The protocol and results are shown in Table 4. Both groups that were pretreated with Cy had more intense challenge reactions; the group given the larger amount of Cy gave the most intense skin reactions.

DISCUSSION

ACD in mice was first produced by Crowle & Crowle (1961); the introduction by Asherson & Ptak (1969) of the ear as a challenge site greatly facilitated its study. ACD of the mouse closely resembles classic ACD of man and of the guinea-pig. The specific effector cell is a T cell. The challenge reactions begin to develop only after 6 or more hours and histologically are characterized by a mononuclear infiltrate (Asherson & Ptak, 1969; Phanuphak, Moorhead & Clayman, 1974). Intravenous pretreatment with the same or a similar hapten results in the induction of specific immunological tolerance (Frei, 1928; Sulzberger, 1929; Asherson, Zembala & Barnes, 1971). In contrast with the guinea-pig, however, in the mouse cellular transfer with unfractionated populations of spleen and lymph node cells is successful only for a few days after the acquisition of sensitivity; indeed, by the ninth day after

Table 4. Immunopotentiation of ACD with different doses of Cy

Group	Treatment Day −3	day 0	Day 7	Increase in ear thickness Day 8	Day 9
(8) I	Cy 150 mg/kg	Oxazolone	Oxazolone	14·9 ± 5·1	13·2 ± 5·9
(8) II	Cy 50 mg/kg	Sensitization	Challenge	11·6 ± 7·0	8·0 ± 5·4
(8) III	—	Sensitization	Challenge	3·6 ± 2·3	1·8 ± 0·9
(8) IV	—	—	Challenge	1·5 ± 1·4	1·9 ± 0·6

Three groups of anti-IgM suppressed mice were sensitized on the flank with 1% oxazolone. In groups I and II, Cy was given 3 days prior to sensitization; group I received 150 mg/kg and group II 50 mg/g. On Day 7, all three groups, as well as a toxicity control group, were challenged on the ear.

The average increases in ear thickness ($mm \times 10^{-2}$), and standard deviations at 1 and 2 days is shown. Using a Mann-Whitney test, at 24 h, group I > III, II > III ($P < 0{\cdot}01$); 48 h, group I > III, II > III ($P < 0{\cdot}01$) and group I > II ($P < 0{\cdot}05$).

sensitization, the spleen and lymph node cells are predominantly suppressive. This has been attributed to B suppressor cells (Zembala *et al.*, 1976). Further ACD in the mouse is relatively transient; by 2–3 weeks after sensitization, it can be demonstrated only irregularly (a similar transience is seen with DTH to SRBC in mice) (Lagrange, Mackaness & Miller, 1974a,b; Askenase, Hayden & Gershon, 1977; Mitsuoka, Teramatsu, Morikawa & Yaswhira, 1978). It may be that the conditions for sensitization induce suppressor cells more readily and more quickly in the mouse than in the guinea-pig.

Cy potentiates DTH to cellular as well as to contact allergens (Easmon & Glynn, 1977; Lagrange, Mackness & Miller, 1974a,b; Kerckhaert, Van den Berg & Hofhuis, 1974). Immunopotentiation of DTH by Cy is not limited to a particular species; it has regularly been demonstrated in guinea-pigs and mice as well as, in our laboratory, in rats, hamsters and chickens. It appears that Cy eliminates a population of suppressor cells that otherwise would dampen the expression of ACD (Polak & Rinck, 1977). In the present experiments we have demonstrated that ACD can be potentiated by Cy in mice that are B-cell depleted by chronic treatment with heterologous antisera to mouse IgM. We found that these immunosuppressed mice lacked serum IgM, had circulating antibody to mouse IgM and failed to form antibody against SRBC. Further, spleen cells from C57Bl/10 mice, which were similarly treated with this same antiserum, made a normal response to the T-cell mitogen phytohaemagglutinin (PHA) but failed to respond to the B-cell mitogen lipopolysaccharide endotoxin (LPS). A number of studies in the literature have attested to the effectiveness of comparable treatment schedules for inducing B-cell suppression. Thus, our study makes it unlikely that a B cell is the target cell of Cy potentiation of ACD. In addition, the ACD reactions of B-cell deficient mice not treated with Cy were equal to those of sensitized intact mice. This implies that B cells do not homeostatically regulate the normal immune response to contact allergens. The same logic works against the point of view that Cy immunopotentiates by inhibiting the formation of suppressive immune complexes (Lagrange, MacKaness & Miller, 1974a,b).

Reactions of ACD have been observed in humans with agammaglobulinaemia as well as in chickens that are B-cell depleted by chemical bursectomy (Weidanz, Weber & Maguire, 1976). Thus, the B cell would appear to be disposable both as a specific and as a non-specific effector cell of allergic contact dermatitis. In our present experiments we found that ACD is well expressed in mice with circulating anti-IgM. This suggests that if the T-cell receptor in ACD is an immunoglobulin, it lacks μ determinants and is not derived from a cell with surface μ determinants. A vigorous rejection of incompatible grafts has been seen in anti-μ suppressed mice (Manning, 1975).

Might the target cell of Cy immunopotentiation be a suppressor macrophage? Since the suppressor cell reactions are specific for the inducing allergen, the suppressor cells must have specific receptors and these receptors almost certainly recognize the carrier specificity of the antigen (Battisto & Chase, 1965). The macrophage itself lacks specific receptors for antigen. Since we have found that Cy immunopotentiation succeeds in the B-cell depleted animal, it is unlikely that specificity could be conferred on the macrophage by cytophilic antibody. Further, consideration of carrier specificity makes it unlikely that cytophilic antibody could confer the necessary specificity to the suppressing macrophage. It is conceivable that suppressor macrophages, which are later 'armed' with specific T cell lymphokine (SMAF), could modulate the acquisition of ACD. Such a subpopulation of suppressor macrophages (prior to their being armed) could be inhibited by Cy. It is much more likely that Cy immunopotentiates as a result of its toxicity for suppressor T cells. Such T cells that suppress DTH may belong to the same population of suppressor T cells that restrain antibody formation; we are currently testing this possibility.

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