Brief Definitive Report

AUGMENTATION OF DELAYED-TYPE HYPERSENSITIVITY BY DOSES OF CYCLOPHOSPHAMIDE WHICH DO NOT AFFECT ANTIBODY RESPONSES*

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Immunization of mice with low doses of sheep red blood cells (SRBC) which are suboptimal for antibody responses, leads to sensitization for delayed foot pad reactions within 4 days (1–3). These delayed-type hypersensitivity (DTH) reactions cannot be elicited in mice which have been immunized with larger doses of SRBC that are more optimal for antibody responses (3, 4). It has been suggested that B-cell responses to high dose immunization cause suppression of T-cell-mediated delayed foot pad reactions (3). In support of this notion, animals pretreated with B-cell depleting doses of cyclophosphamide (2–300 mg/kg) have markedly suppressed antibody responses and augmented delayed reactions (4–7). We have studied delayed foot pad reactions of mice immunized with SRBC and found that by lowering the dose of cyclophosphamide pretreatment to 20 mg/kg, delayed-type hypersensitivity can be augmented without affecting antibody responses. Thus antibody is not the sole regulatory factor in mice immunized with high doses of SRBC.

Materials and Methods

Groups of five to six male mice (5 wk old) were obtained from Jackson Laboratories (Bar Harbor, Maine), and were immunized intravenously with 0.2 ml of different concentrations of washed SRBC 1 wk after arrival. Cyclophosphamide (Cytoxan, Mead Johnson & Co., Evansville, Ind.) was dissolved in sterile saline immediately before use and injected by the intraperitoneal route as a single dose of 20 mg/kg 1 day before immunization, or 200 mg/kg 2 days before immunization.

4 days after immunization, one foot pad per mouse was measured for thickness with a micrometer (Brown & Sharps Mfg. Co., No. Kingston, R.I.) and was then tested by injection of 0.03 ml of 20% SRBC in sterile saline. The original foot pad thickness was compared to subsequent measurements to compute the percent increase in thickness. Animals were re-tested in the contralateral foot pad 10 days after immunization. Blood samples were obtained by capillary tube puncture of the retro-orbital plexus 5 and 10 days after initial immunization. The resulting serum from individual mice was twofold diluted in 0.025-ml volumes in microtray wells for measurement of hemagglutinating antibody (HA) titers. Student's t test was used to compute statistical significance ($P < 0.05$) of HA titers and DTH.

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Results

The data (Table I) show that in BDP mice a low dose of immunizing antigen (0.01% SRBC: about 2×10^5 erythrocytes), produced a much greater DTH response than did a larger dose (1% SRBC) which was more optimal for antibody production. (These delayed foot pad reactions achieved with low dose SRBC immunization were maximal in these mice at 48 h, were specific for SRBC compared to equivalent testing with horse, goose, or human erythrocytes; were unaccompanied by significant 4-h reactions; and could be transferred to nonimmune mice with spleen cells and not sera [data not presented].) Cyclophosphamide pretreatment in low doses (20 mg/kg) had little effect on delayed foot pad reactions and hemagglutinating antibody production in the mice immunized intravenously with 0.01% SRBC. On the other hand, this cyclophosphamide pretreatment administered to mice primed with 100-fold higher doses of SRBC, markedly augmented the small DTH reactions, while vigorous IgG antibody responses were unaffected or slightly increased. Other strains of mice varied in the optimal SRBC dosage for DTH, and the onset and duration of these reactions; but the ability of low dose cyclophosphamide to augment DTH without affecting antibody responses was found in 10 other strains of mice (AKR, DBA/2, B6AF$_1$, AKD2F$_1$, C57BL/6, BDF$_1$, A, BALB-B, C3H/NB, and NZB). The BDP mice shown in Table I were selected from various strains enumerated above as they best illustrated the general phenomenon.

TABLE I

Augmentation of Delayed-Type Hypersensitivity in Mice, by Doses of Cyclophosphamide Which Do Not Affect Antibody Responses

Immunizing dose of SRBC (0.2 ml)	Cyclophosphamide pretreatment	Responses after foot pad testing at 4 days		
		48-h thickness (% change ± SE)	24-h HA titer $\log_2$	
			Plain	+ 2 ME
0.01%	0	43 ± 3	1 ± 0.4	1 ± 0
0.01%	20 mg/kg	43 ± 6	1.3 ± 0.5	1 ± 0
1.0%	0	15 ± 1	5.3 ± 0.5	4.5 ± 1.2
1.0%	20 mg/kg	50 ± 2*	6.3 ± 0.5	6.3 ± 0.5

* $P \ll 0.0005$ compared to equivalently immunized controls that were not pretreated with cyclophosphamide.

Table II shows the results of an experiment in which the immunizing dose of SRBC and pretreatment dose of cyclophosphamide were varied in BDF$_1$ mice and foot pad testing performed on day 4 and on day 10. As with BDP mice, immunization with 0.01% SRBC led to optimal DTH reactions and negligible 4-day antibody responses. Immunization with supraoptimal SRBC doses (1% and 100%) led to suppressed DTH reactions, which were augmented by low dose cyclophosphamide pretreatment without affecting the large antibody responses. When mice immunized with the higher of these doses of SRBC were pretreated with higher doses of cyclophosphamide (200 mg/kg), the antibody responses were abolished but no further augmentation of DTH occured. Thus, the augmentation of delayed hypersensitivity by cyclophosphamide pretreatment was largely independent of its effect on antibody responses. Furthermore, mice immunized

TABLE II

The Effect of Cyclophosphamide Pretreatment on Delayed Hypersensitivity and Antibody Responses in BDF_1 Mice 4 and 10 Days after Intravenous Immunization with Various Doses of SRBC

Immunizing dose of SRBC	Cyclophosphamide pretreatment	Responses after foot Pad testing			
		At 4 days		At 10 days	
		DTH*	HA titer	DTH*	HA titer
		%	log_2	%	log_2
0.001%	0	14 ± 2	0	13 ± 4	6.8 ± 0.3
0.001%	20 mg/kg	41 ± 7‡	0	23 ± 5	7.3 ± 0.3
0.01%	0	60 ± 7	0	32 ± 3	7.8 ± 0.2
0.01%	20 mg/kg	54 ± 4	0.8	28 ± 2	8.5 ± 0.2
1.0%	0	−2 ± 3	6.5 ± 0.2	11 ± 3	7.6 ± 0.2
1.0%	20 mg/kg	14 ± 5‡	6.5 ± 0.2	10 ± 3	7.5 ± 0.3
1.0%	200 mg/kg	13 ± 2‡	0	46 ± 5‡	2.4 ± 0.7‡
100%	0	4 ± 3	6.2 ± 0.5	5 ± 1	8.3 ± 0.2
100%	20 mg/kg	10 ± 3	5.2 ± 0.3	5 ± 1	8 ± 0
100%	200 mg/kg	15 ± 2‡	0.7	34 ± 2‡	0‡

* Percent increase in footpad thickness at 24 h ± SE.
‡ $P < 0.005$ compared to equivalently immunized controls that were not pretreated with cyclophosphamide.

with lower than optimal doses of SRBC (0.001 %), produced no detectable antibody response at 4 days and also had augmented DTH responses when pretreated with 20 mg/kg cyclophosphamide. Thus, DTH responses which were suppressed by either high zone or low zone immunization with SRBC, could be augmented by cyclophosphamide without affecting antibody responses.

On day 10, DTH reactions were still present in mice immunized with low doses of SRBC but were decidedly less than reactions elicited at 4 days, and were accompanied by appreciable antibody titers. Augmented DTH reactions were now found principally in mice treated with the higher doses of cyclophosphamide and antigen, and were associated with greatly depressed mean antibody titers. Thus, at day 10 in contrast to day 4, augmented DTH by cyclophosphamide correlated with depressed antibody responses. However, examination of the individual mice which had augmented DTH reactions on day 10 showed that augmentation was as great in those nine mice with antibody (36 ± 3% DTH; HA titer 4 ± 0.4) as it was in the 12 mice with absent antibody responses (39 ± 4% DTH). Thus, even the augmented DTH produced by greater suppressive doses of cyclophosphamide was not correlated with the degree of suppression of the antibody response.

Discussion

We have shown in a variety of mouse strains that pretreatment with a very small dose of cyclophosphamide can augment the delayed reactions elicited by sheep cell injection in the foot pads of mice immunized with greater than optimal doses of antigen. These small doses of cyclophosphamide have no detectable suppressive effect on the production of hemagglutinating antibodies at the time of

testing for delayed sensitivity. If cyclophosphamide augments the DTH response by removing a suppressor influence, it would appear that antibody is not the sole suppressor of these reactions. Other workers have shown that higher doses of cyclophosphamide also potentiate delayed reactions and concomitantly depress antibody responses. Our work suggests that the association of these two effects of cyclophosphamide are not necessarily causatively related. In fact, in several instances delayed foot pad reactions and antibody responses were both augmented by cyclophosphamide pretreatment. In addition, mice immunized with suboptimal doses of SRBC produced no detectable antibody at 4 days and also had DTH that was augmented by cyclophosphamide. It is intriguing to speculate that this low zone suppression of DTH, which was uncovered by cyclophosphamide pretreatment, is related to low zone tolerance for antibody production.

A number of manipulations produce reciprocal effects on antibody formation and delayed hypersensitivity. In particular, acetoacetylation or periodination of flagellin or SRBC (8, 9), hapten conjugation of proteins (10) and glutaraldehyde pretreatment (11), or macrophage preincubation of SRBC (12) augments delayed reactions and suppresses antibody responses. These modifications of antigen may mimic the effects produced by decreasing antigen doses as employed in this study. Mice immunized with 100% SRBC were particularly intriguing; pretreatment of these mice with 200 mg/kg of cyclophosphamide, similar to antigen modification, led to complete antibody tolerance (the mice made no antibody at day 10 after priming on day 0 and challenge on day 4) although good DTH responses could be elicited.

It is now quite well established that T cells can have both augmentative and suppressive effects on other T cells as well as on B cells, (reviewed in 13). Such T cells can be thought of as regulators of the immune response. It is possible that antigen dose and/or other molecular factors may induce regulatory T cells to produce one type of immunity and concomitantly suppress another. Doses of antigen which are optimal for antibody production by B cells may induce regulatory T cells to suppress delayed hypersensitivity responses independently of the ensuing antibody response. Thus, the frequently noted reciprocal relationship between antibody formation and delayed hypersensitivity may be, at least in part, regulated by T cells directly, without indirect participation of feedback signals from target cells (i.e. antibody).

B-cell products may play an additional role in suppression of delayed hypersensitivity since it is well known that antibody can be immunosuppressive. B-cell products could act directly to suppress delayed hypersensitivity or could act to signal regulatory T cells to exert suppressor effects; as has been recently reported (14,15).

Although initial reports indicated that cyclophosphamide selectively depleted nonthymus-dependent areas of lymphoid tissues, depressed antibody responses, and was not toxic for T cells, more recent information suggests that subpopulations of T cells are rapidly dividing (16) and thus probably sensitive to cyclophosphamide (17).

Although low dose cyclophosphamide pretreatment failed to produce reductions in hemagglutinating antibody titers, subpopulations of B cells producing antibodies of specialized classes or other B-cells products, could have been af-

fected. One must keep open the possibility that small quantities or local production of some B-cell products may be particularly specialized to act as regulatory molecules. Further studies are required to rule out this possibility. However, it is quite clear from the results we have presented that gross changes in the delayed hypersensitivity response can be produced by cyclophosphamide pretreatment without the production of concomitant gross alterations in the antibody response.

Summary

Mice immunized with more SRBC than are required to produce optimal delayed-type hypersensitivity reactions, developed good antibody responses and poor delayed foot pad reactions. Cyclophosphamide treatment in low doses (20 mg/kg) before immunization, augmented the delayed-type hypersensitivity without affecting antibody responses. Cyclophosphamide did not augment delayed responses to optimal doses of SRBC (0.01%), but did augment the delayed hypersensitivity response of mice immunized with a suboptimal antigen dose (0.001%); which produced no detectable antibody response with or without cyclophosphamide pretreatment. These results suggest that antibody feedback is not the sole regulator of delayed reactions; the possibility that suppressor T cells may also be involved is discussed.

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