SELECTIVE ABROGATION OF ANTIGEN-SPECIFIC HUMAN B CELL RESPONSES BY ANTIGEN-RICIN CONJUGATES

By DAVID J. VOLKMAN, ATEEQ AHMAD, ANTHONY S. FAUCI, AND DAVID M. NEVILLE, JR.

From the Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, and Section on Biophysical Chemistry, Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, Maryland 20205

The selective elimination of subpopulations of cells on the basis of their capacity to specifically bind various toxin conjugates has been the focus of recent intensive interest and study (1, 2). The regulatory mechanisms that control the specificity and size of discrete B lymphocyte clones occasionally break down and results in the production of deleterious antibodies directed against self determinants. In such cases, it would be desirable to suppress specific B cell clones without inhibiting the normal functions of the remainder of the immune system. Using an in vitro model system of antigen-specific antibody synthesis (3, 4) and an antigen-toxin conjugate, we demonstrate that selective abrogation of antigen-specific human B cell responses can be achieved.

The rationale for the procedure of using a toxin-conjugate to reduce the population of specific cells has been previously outlined (1). Toxins such as ricin and diphtheria consist of two chains, each having different functions (5, 6). The A chain is an enzyme capable of inactivating protein synthesis once it is transported to the cytosol compartment. The B chain initiates the entry process by binding the toxin to cell surface receptors present on most cell types. By replacing the normal toxin B chain with a new binding chain having receptor specificity for a distinct cell type, a structural analogue of the toxin is created (1). The specificity of cell killing is now determined by the new binding chain. A variety of such toxin analogues have been constructed with varying degrees of potency and cell type specificity (7, 8).

The antigen-toxin conjugate used in these studies is a thioether conjugate of tetanus toxoid (TT) and intact ricin containing both the A and B chains. This was done for the initial studies because of recent evidence showing that the toxin-binding B chain plays a role in facilitating A chain entry (9). This role appears to be independent of surface membrane receptor binding (2, 10). Certain conjugates containing toxin B chains have been shown to have faster rates of specific cell killing (2, 9). Conjugates of ricin containing the B chain are used in the presence of lactose, which largely (20-200-fold) blocks the ricin entry route via its receptor and thereby reduces nontarget cell killing (9). Conjugates constructed along these lines, such as mannose-6-PO₄-ricin and monoclonal anti-Thy-1.2-ricin, have been useful in selecting mutant cell lines deficient in mannose-6-PO₄ receptors (11) and in eliminating T cells from murine bone marrow allografts as a means of preventing experimental graft-vs.-host disease (12), respectively. In both of these cases, the selectivity of the conjugate between target and nontarget cells is between 100:1 and 1000:1, and the desired objective is achievable without complete elimination of the target cell population (11, 12).
B lymphocytes have surface immunoglobulin (Ig) that specifically binds to the antigenic determinant (Ag) against which the B cell is capable of producing antibody. This Ag-specific surface antibody represents a sensitive Ag-recognition mechanism and confers a high degree of specificity and affinity to the binding of Ag by Ag-specific B cells, thus providing a potential method for the selective elimination of these specific B cells via an Ag-ricin conjugate, while leaving intact the remainder of the B cell repertoire.

Materials and Methods

**Immunization.** Normal subjects, aged 18-25 yr, received a standard booster dose of 5 Lf units of tetanus toxoid intramuscularly (Wyeth Laboratories, Marietta, PA). Certain subjects had also been previously immunized with two subcutaneous injections of 5 mg of keyhole limpet hemocyanin (KLH) spaced 2 wk apart.

**Cell Separations.** Human peripheral blood mononuclear cells (PBMC) were obtained from either heparinized whole blood or buffy coat fractions by sedimentation over Ficoll-Hypaque gradients in a standard fashion (13). In certain experiments where indicated, T cells were removed from these mononuclear cell fractions by treatment with the anti-T cell monoclonal antibody Leu-1 (Becton, Dickinson & Co., Oxnard, CA) and rabbit complement (Dutchland Laboratories, Inc., Denver, PA) for 1 h at 37°C, as previously described (4).

**Antigens.** TT for use in vitro was generously provided by Lederle Laboratories, Pearl River, NY. The toxoid was further purified by chromatography on Sephacryl S-200 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) into a homogenous molecular weight fraction of 150,000. The antigenicity of this material was identical to that of the crude toxoid as determined by an enzyme-linked immunosorbent assay (ELISA) (3).

**Conjugate Synthesis.** Thiolated tetanus toxoid was conjugated with ricin via a thioether linkage. To 5 mg of purified toxoid in 1 ml of buffer, 0.1 M NaPi, pH 7.2, 100 µg of 20 mM N-succinimidyl-3(2-pyridyldithio)-propionate (SPDP) in ethanol was added with stirring. After 30 min at 25°C, 75 mg of dithiothreitol was added. The thiolated toxoid was freed of reducing agent by running over a 1.8- x 15-cm Sephadex G25F column in the same buffer. Ricin D, 3.5 mg in 0.2 ml isolated and derivatized with m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), as previously described (14), was added to the peak fraction of the thiolated toxoid. After 3 h, the reaction was terminated by adding 50 µl of 1 M N-ethylmaleimide. The reaction mixture was chromatographed at 1 ml/min in the above buffer by high-pressure liquid chromatography on a 7.5- x 600-mm SW TSK 3000 column. A peak at 12.5 ml was observed as well as unreacted ricin at 19 ml. Thiolated toxoid ran at 13.5 ml. Fractions 8-14.5 ml were pooled, applied to a 2-ml Sepharose 4B column at 4°C. Washing with phosphate-buffered saline (PBS) removed unreacted tetanus toxoid, while ricin conjugate remained bound. The conjugate was eluted as a sharp peak with 100 mM lactose in PBS.

**Culture Conditions.** Cells were preincubated with various combinations of TT, ricin, and/or TT-ricin conjugates for 2 h at 37°C in RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 100 mM lactose to inhibit binding of ricin to nontarget cells. Preincubation was performed at a cell density of 1-2 x 10^6 cells in 3 ml of medium. Cells were then washed once with a 25 mM lactose RPMI 1640 solution and then twice more with RPMI 1640 alone. After preincubation, cells were cultured in 12- x 75-mm round-bottomed tubes at between 5 x 10^6 and 8 x 10^6 cells per tube for 10 d in 1 ml of RPMI 1640 containing 10% heat-inactivated fetal calf serum, 20 µg/ml gentamicin (Schering-Plough Corp., Kenilworth, NJ), and the polyclonal B cell activator pokeweed mitogen (PWM) at a final dilution of 1/200 of the stock solution Gibco Laboratories, Grand Island Biological Co.)

Cultures for measuring blast transformation were performed as previously described (4). Cells were stimulated with TT, concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO), phytohemagglutinin (Burroughs-Wellcome, Beckenham, England), or KLH for 5 d at 37°C in 5% CO_2, then pulsed with 0.4 µCi [³H]thymidine for 4 h, harvested on a Titertek cell harvester (Flow Laboratories, Inc., McLean, VA) and counted in a scintillation counter.

**Ig Assays.** Supernatants were assayed for anti-TT IgG, anti-KLH, IgM, and total IgG and IgM, as previously described (3, 4), using an ELISA system using heavy chain-specific goat anti-human IgG or IgM conjugated to the enzyme alkaline phosphatase (Sigma Chemical Co.).
Results

The effect of preincubation of PBMC with TT-ricin on subsequent PWM-stimulated Ig production was examined. Fig. 1 shows a representative experiment in which PBMC were obtained from an individual recently immunized to TT. Preincubation with TT alone or with a mixture of TT and ricin had virtually no effect on subsequent anti-TT or total Ig production. However, brief exposure to TT covalently linked to ricin at concentrations >10 ng/ml abrogated anti-TT synthesis ($P < 0.01$) but did not diminish total Ig production. TT-ricin also selectively eliminated anti-TT antibody production without decreasing total Ig production in PBMC from three additional TT-immunized individuals. One of these subjects was immunized to both TT and KLH. In this case, preincubation with the TT-ricin conjugate eliminated PWM-induced anti-TT production but did not affect either anti-KLH or total Ig production. Thus, TT-ricin selectively removed anti-TT antibody synthesis but was not toxic to B cells specific for an unrelated antigen (KLH). Preincubations were performed in the presence of 100 mM lactose to prevent binding of ricin conjugates to cells via their membrane galactose molecules. Preincubation with unconjugated ricin at up to 100 ng/ml under these conditions did not affect anti-TT or total Ig production. When lactose was not present, TT-ricin at 1 ng/ml inhibited PWM-induced anti-TT antibody synthesis as well as total Ig synthesis by 90%. Ricin alone showed total inhibition of these responses at 0.5 ng/ml (data not shown).

The target cell of TT-ricin action was studied by preincubating T cell-depleted B cells with TT-ricin conjugate. Cells were preincubated at various concentrations of either TT alone (○), TT plus free ricin (□), or TT-ricin conjugate (○) for 2 h at 37°C in the presence of 100 mM lactose, as described in Materials and Methods. Data are expressed as the geometric mean of triplicate determinations of either anti-TT IgG production (A) or total IgG production (B) of 10-d culture supernatants. ○, TT alone; ○, TT-ricin; □, TT plus ricin.

Fig. 2. Selective abrogation of anti-TT production in B cells after preincubation with TT-ricin conjugate. Unfractionated PBMC (○, ○) or B cells (□, □) were preincubated with either TT alone (○, □) or TT-ricin conjugate (○, □) for 2 h at 37°C in the presence of 100 mM lactose. Cultures of either $5 \times 10^5$ preincubated unfractionated cells or $2 \times 10^5$ preincubated B cells plus $5 \times 10^5$ fresh irradiated unfractionated cells (1,500 rad) were incubated for 10 d, and supernatant anti-TT IgG or total IgG were assayed. Data represent the geometric mean of triplicate cultures assayed. ○, UF (TT alone); ○, UF (TT-ricin); □, B (TT alone); □, B (TT-ricin).
TABLE I

Effect of TT-ricin Pretreatment on Subsequent Proliferative Responses of Human Mononuclear Cells

| Pretreatment* | Subsequent stimulation‡ |
|---------------|-------------------------|
|               | None       | TT (10 µg/ml) | TT (20 µg/ml) | Con A (100 µg/ml) | KLH (20 µg/ml) | PHA (2 µg/ml) |
| No antigen    | 861§       | 30,076       | 38,763       | 13,404           | 19,617        | 20,388        |
| (± 20)        | (± 2,219)  | (± 2,081)    | (± 1,198)    | (± 2,940)        | (± 1,494)     |
| 10 ng TT      | 189        | 29,381       | 34,289       | 9,421            | 19,093        | 25,441        |
| (± 30)        | (± 4,431)  | (± 3,119)    | (± 1,773)    | (± 1,742)        | (± 1,223)     |
| 100 ng TT     | 398        | 31,450       | 29,984       | 10,714           | 21,224        | 28,740        |
| (± 35)        | (± 1,819)  | (± 1,737)    | (± 1,039)    | (± 2,468)        | (± 1,282)     |
| 100 ng TT-ricin| 384       | 27,005       | 39,223       | 8,368            | 23,306        | 20,478        |
| (± 56)        | (± 566)    | (± 4,652)    | (± 1,020)    | (± 4,231)        | (± 1,923)     |
| 100 ng TT-ricin| 1,623     | 13,320       | 14,967       | 5,973            | 13,839        | 21,854        |
| (± 103)       | (± 189)    | (± 1,213)    | (± 143)      | (± 1,451)        | (± 531)       |

* PBMNC were preincubated in 100 mM lactose, RPMI 1640 with the designated antigen for 2 h at 37°C, then washed as described in Materials and Methods.
‡ 10^6 cells in 200 ml were cultured in round-bottomed microtiter wells for 5 d and then pulsed with 0.4 µCi [3H]thymidine for 4 h and harvested.
§ Data represent mean cpm of triplicate determinations (± SEM).

Discussion

The present study demonstrated that human antigen-specific B cell responses can be selectively eliminated by pretreatment in vitro with antigen linked to the cell toxin ricin. The remainder of the B cell repertoire, as indicated by polyclonal Ig responses and antibody responses directed against other non-cross-reacting antigens, is preserved. The lack of significant effect of TT-ricin exposure on T cell proliferative responses to subsequent TT stimulation as well as the fact that preincubation of purified B cells with TT-ricin selectively abrogated the anti-TT antibody responses of these B cells, strongly suggest that TT-ricin conjugates bind directly to the TT-specific B cells via their specific surface Ig receptors.

In the present study, ricin B chain was left attached to the A chain in making the ricin-TT conjugates. The B chain of ricin binds to virtually all mammalian cells via glycoprotein galactose residues and also facilitates entry of the toxic A chain into cell (2, 9, 10). Because lactose competitively inhibits this binding of the ricin B chain to cell fractions with either TT alone or TT-ricin conjugate (Fig. 2). After preincubation, these cells were reconstituted with irradiated unfractioned mononuclear cells, and PWM-induced Ig synthesis was assessed. Preincubation with TT-ricin eliminated anti-TT antibody production in both unfractionated cells and in the B cell fraction (P < 0.01), whereas pretreatment with unconjugated TT did not abrogate anti-TT responses. Again, total Ig production was not diminished by TT-ricin pretreatment.

On the other hand, pretreatment of PBMC with TT-ricin did not abolish T cell blastogenic responses to TT. Table I shows the results of preincubation of unfractionated cells with TT alone or TT-ricin in the presence of 100 mM lactose and subsequent stimulation. At concentrations of TT-ricin shown to block anti-TT antibody production in both unfractionated cells and in the B cell fraction (P < 0.01), whereas pretreatment with unconjugated TT did not abrogate anti-TT responses. Again, total Ig production was not diminished by TT-ricin pretreatment.

These results, taken together, indicate that TT-ricin is capable of selectively eliminating TT-specific B cells without significantly affecting the remainder of the B cell repertoire or the T cell population.

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cell surfaces (2), preincubations were performed in the presence of 100 mM lactose to inhibit binding of the B chain containing complex to nontarget cells. Removal of the B chain from the TT-ricin conjugate markedly decreases the nontarget cell toxicity of the compound and precludes the necessity of incubation in lactose and thus opens the possibility of in vivo use of these conjugates. In this regard, preliminary in vitro studies with TT-ricin A chain conjugates alone have demonstrated similar selective abrogation of anti-TT antibody responses (unpublished observations).

Previous studies (8, 12, 14, 15) demonstrated the feasibility of eliminating populations of cells by attaching toxins of various types to antibodies that recognize specific antigenic determinants of lymphocyte surfaces. In those studies, the specificity of the binding was at the level of the conjugated antibody directed against a cell surface determinant and was not directly related to the functional capability of the target cell. In the present study, the specificity and selectivity of the cytotoxicity is directly related to the functional capability of the cell being eliminated, i.e., its surface Ig or antigen recognition moiety. There is obvious therapeutic potential in eliminating B cells by virtue of their antigen-binding capability in diseases characterized by aberrant antibody responses, such as the antibody-mediated autoimmune diseases in which the antigen has been identified (16, 17). Examples of such diseases include systemic lupus erythematosus nephritis mediated by anti-DNA antibodies, Goodpasture's disease mediated by anti-basement membrane antibodies, myasthenia gravis mediated by anti-acetylcholine receptor antibodies, and several autoimmune endocrine and hematologic diseases (17). The non-neoplastic nature of these diseases might allow clinically relevant suppression of disease activity by such an approach because abrogation of a significant proportion but not the entire autoimmune response might be sufficient to render the disease subclinical. This has the distinct advantage over the treatment of malignant tumors with antibody-toxin conjugates because in animal models complete elimination of viable tumor cells must be achieved to prevent recurrences (15).

Summary

The feasibility of selectively eliminating human antigen-specific B cell responses by treating cells in vitro with antigen covalently linked to a cell toxin was examined. Tetanus toxoid (TT) was conjugated to the toxin ricin via a thioether linkage. Peripheral blood mononuclear cells from recently immunized subjects were preincubated for 2 h with TT-ricin in the presence of lactose. Antigen was then removed, and the cells from recently immunized subjects were preincubated for 2 h with TT-ricin in the presence of lactose. Antigen was then removed, and the cells were stimulated with pokeweed mitogen to induce antibody production. TT-specific antibody production was completely abrogated by preincubation with TT-ricin but not by TT alone or a mixture of TT and ricin. In contrast, polyclonal immunoglobulin production was not diminished by TT-ricin. This selective abrogation was also seen when B cells alone were preincubated with TT-ricin and a source of T cell help was later provided. T cell blastogenic responses to TT remained intact after TT-ricin exposure. Thus, antigen-toxin conjugates are capable of selectively eliminating specific antibody-producing B cell clones, while leaving intact the remainder of the B cell repertoire.

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