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Research Article

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Selective Elimination of Anti-DNA Antibody-producing Cells by Antiidiotypic Antibody Conjugated with Neocarzinostatin

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Abstract

A new strategy was shown for the manipulation of autoantibody production in humans. Antiidiotypic antibody to human anti-DNA autoantibody was conjugated with neocarzinostatin (NCS), a cytotoxic agent, by using *N*-succimidyl 3-(2-pyridyldithio) propionate as a coupling agent. Human B cell clones, which produce anti-DNA autoantibodies, were killed by in vitro treatment with antiidiotype (Id)-NCS conjugates, while clones expressing an Id with irrelevant specificity were unaffected. These results indicate that treatment with anti-Id–NCS conjugates can act as a potent and specific means of generating immunosuppression of autoantibody production. This approach will have a significant advantage in aborting clones that are not effectively suppressed for the autoantibodies by anti-Id antibodies alone, and will result in a potential therapeutic treatment for systemic lupus erythematosus.

Introduction

There is a need for a new therapeutic approach for autoimmune diseases, since therapy is as yet unsatisfactory. Some autoantibodies have been believed to play a key role in the pathogenesis of immunological disorders (1, 2). These observations have led to the realization that the specific manipulation of the production of autoantibodies, which contribute to the formation of lesions in systemic lupus erythematosus (SLE),[1] would be one recommended approach to therapy (3, 4). In this respect, the use of antiidiotypic (Id) antibody might be an acceptable candidate for the in vivo regulation of autoimmune disorders (5–7). Anti-Id antibodies can not only induce the suppression of the in vitro production of rheumatoid factors, but also improve the prognosis of NZB/W F_1 mice (8, 9). Anti-Id immunity, however, failed to suppress an already established spontaneous antibody production (10), and in another system the administration of anti-Id antibody elicited the production of antibodies to thyroglobulin (11). This variety in the results may be due to the complexity in anti-Id immunity, which includes a direct action to B cells and an activation of T cell circuits (12, 13). It would therefore be desirable to develop a strategy in which the clones producing autoantibodies were specifically and strongly eliminated.

We established human B cell clones producing monoclonal anti-DNA autoantibodies (14), which serve as useful models for anti-DNA antibody-producing cells in human SLE. Since anti-Id antibody to human anti-DNA antibody can specifically bind these clones (15), anti-Id antibody in combination with a cytotoxic agent might be expected to selectively kill the Id-positive cells. Therefore we have developed a new procedure for the specific manipulation of autoantibody production, using anti-Id antibody coupled to neocarzinostatin (NCS).

Methods

Antibodies. Human monoclonal anti–double-stranded (ds) DNA or anti–single-stranded (ss) DNA antibodies were obtained from NE-1 or O-81 clones, which are B cell clones transformed by Epstein-Barr virus (EBV) infection (16). Hybridoma-secreting anti-Id antibodies were produced by the fusion of a nonsecreting myeloma cell line, SP 2/0, with spleen cells of Balb/c mice immunized with affinity-purified monoclonal anti-DNA antibodies (0-81 or NE-1) according to the technique of Köhler and Milstein (16a). Anti-Id antibodies to O-81 and NE-1 were designated as D1E2 and 1F5, respectively. 1F5 had a restricted specificity for surface Id on anti-ds DNA clones (NE-1), whereas D1E2 bound to the surface Id on anti-ss DNA clones (O-81), but not to those on anti-ds DNA clones. The anti-Id antibodies were purified twice using a DEAE-cellulose column as IgG.

Preparation of anti-Id-conjugated NCS. Purified NCS (5 mg/ml, Kayaku Antibiotics Research Co. Ltd., Tokyo, Japan) was first incubated with a fourfold molar excess of *N*-succinidyl 3-(2-pyridyldithio) propionate (SPDP, Pharmacia Fine Chemicals, Uppsala, Sweden) in a 0.1 M phosphate buffer, pH 6.5, at 25°C for 30 min. The 3-(2-pyridyldithio)-propionylated (PDP) NCS was reduced with 10 mM dithiothreitol (DTT, Wako Pure Chemicals Industries, Tokyo, Japan) in a 0.1 M acetate buffer, pH 4.5, at 25°C for 30 min. The resulting thiol groups–introduced NCS, HS-NCS, was immediately passed through a Sephadex G-25 column. Anti-Id antibody to DNA antibody (2.8 mg/ml) or control mouse IgG was incubated with a 10-fold molar excess of SPDP in a 0.1 M phosphate buffer, pH 6.5, at 25°C for 30 min, and PDP antibody was purified by gel filtration on a Sephadex G-25 column. Finally, PDP antibody was mixed with a sixfold molar excess of HS-NCS (thiol groups introduced), and allowed to stand at 25°C overnight in the dark. The mixture was then applied to a Sephacryl S-200 column equilibrated with phosphate-buffered saline (PBS), pH 6.0, and eluted with the same buffer. The peak fractions were pooled, concentrated, and used as anti-Id–NCS. NCS activity of the conjugate preparation was measured by an agar dilution method using Micrococcus luteus ATCC 9341 as the sensitive micro-

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1. *Abbreviations used in this paper:* ds, double-stranded; DTT, dithiothreitol; EBV, Epstein-Barr virus; Id, idiotype; IF, immunofluorescent examination; NCS, neocarzinostatin; PDP, 3-(2-pyridyldithio)-propionylated; SLE, systemic lupus erythematosus; SPDP, *N*-succinidyl 3-(2-pyridyldithio) propionate; ss, single-stranded.

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organism. The extent of substitution in this preparation was ~1 mol/NCS per mol IgG.

Cell culture and assay for cytotoxic activity. Human B cell clones producing monoclonal anti-ds DNA antibody (NE-1, NE-13, NE-28, and NE-29) and anti-ss DNA antibody (O-81) were maintained in RPMI-1640 with 20% fetal calf serum (Gibco, Grand Island, NY) containing 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM L-glutamin. 2 d after the subculture, cells were harvested and washed in RPMI-1640 and 5×10^5 cells in media were placed into round tubes. Then, the anti-Id–conjugated NCS at appropriate dilution in media was added to triplicate culture at one-tenth the volume of the media. After incubation at 37°C, the numbers of viable cells were determined by the trypan blue dye exclusion method. The viability determined by this method was used as a parameter for cytocidal activity. In one experiment the cells were washed 6 h after inoculation with anti-Id–NCS. After removing anti-Id–NCS by washing three times with RPMI-1640, the cells were again incubated at 37°C in 5% CO_2. The viability of the cells and DNA-binding capacity in the culture supernatants were then checked.

Measurement of anti-DNA and anti-Id activity. Anti-DNA activity was sought by a solid-phase radioimmunoassay (RIA) as described previously (4). In short, the culture supernatant was incubated in the wells coated with ss-DNA for 90 min at room temperature. After the cells were washed three times, 100 μl of ^{125}I-labeled affinity-purified anti-human Ig F(ab')$_2$ (2×10^4 cpm) containing 1% bovine serum albumin (BSA) and 0.01% Tween-20 was added, and again incubated at room temperature for 2 h. After three washings with PBS, the radioactivity was measured in a γ-counter. For the measurement of anti-Id activity to anti-DNA antibody, a competitive inhibition assay was performed. Monoclonal anti-DNA antibodies (50 μl) at an appropriate dilution in PBS containing 0.2% BSA and 0.01% Tween-20 were mixed with 50 μl of anti-Id in wells coated with ss-DNA or ds-DNA and incubated for 90 min at room temperature. After washing, their ability to bind to ^{125}I-labeled anti-human μm F(ab')$_2$ (2×10^4 cpm) was tested as described above.

Detection of surface of Id-positive cells. Immunofluorescent staining of surface Id was performed by a two-step method: Cells (1×10^6) were suspended in 100 μl of anti-Id antibodies (D1E2 and/or 1F5) at a 1:100 dilution at 4°C. After washing three times with cold PBS, the cells were again incubated with fluorescein-labeled anti-mouse IgG F(ab')$_2$ (Jackson Laboratory, CA) on ice for 30 min. The cells were again washed three times and suspended in PBS for a flow cytometry analysis (ABCASS, CS-20, Schowa Denko, Tokyo, Japan) or in PBS-glycerol 1:1 for immunofluorescent examination (IF).

Results

Properties of anti-Id–NCS. The prepared anti-Id–NCS formed precipitin lines with both anti-mouse Ig antisera and anti-NCS sera in immunodiffusion test; thus, NCS was successfully conjugated to the anti-Id antibody. The relevant features of each conjugate used in this study are summarized in Table I. D1E2-NCS or 1F5-NCS retained both drug and anti-Id antibody activity in terms of the binding capacity to human monoclonal anti-DNA antibody and of the inhibitory ability on DNA binding of anti-DNA antibodies. IF revealed that each conjugate also specifically reacted with either O-81– or NE-1–human monoclonal anti-DNA antibody-producing clones.

Inhibitory effect of NCS-conjugated anti-Id antibody on the proliferation of anti-DNA–producing cells. Both anti-Id–NCS conjugates were tested for their specific cytotoxicity. First, NE-1 cells producing monoclonal anti-ds DNA antibody were cultured with anti-Id–NCS, anti-Id alone, or mouse IgG-NCS. Anti-Id antibody alone was not toxic at any concentration, whereas all the conjugates including mouse IgG-NCS were cytotoxic in a dose-related manner. The treatment of NE-1 cells with 1F5-NCS showed an especially marked toxicity to the cells, even at concentrations at which other conjugates were no longer cytotoxic (data not shown). NE-1 cells were then incubated with each conjugate for 6 h. After washing, the cells were further cultured at 37°C and the culture was tested for viability and DNA-binding capacity. Fig. 1 shows that 1F5-NCS inhibited the growth of the clones, while they were unaffected by D1E2-NCS, mouse IgG-NCS, and anti-Id alone. The cytotoxicity tests revealed the actual killing of NE-1 clones by 1F5-NCS, whereas most of the cells were alive but inhibited to grow at the higher concentration of D1E2-NCS or mouse IgG-NCS. Fig. 2 also

Table I. Anti-Id Activity in the Conjugates

	Inhibitory activity to DNA binding of		Direct binding ability to	
	O-81 Ab*	NE-1 Ab*	O-81 clones‡	NE-1 clones‡
D1E2	1:102,400	<1:10	1:2,500	<1:10
D1E2-NCS	1:25,800	<1:10	1:2,000	<1:10
1F5	<1:10	1:51,200	<1:10	1:2,000
1F5-NCS	<1:10	1:25,800	<1:10	1:2,000
Mouse Ig	<1:10	<1:10	<1:10	<1:10
Mouse Ig-NCS	<1:10	<1:10	<1:10	<1:10

* The maximum dilution of each reagent was expressed for the inhibitory activity of O-81 or NE-1 binding to DNA as described in Methods.

‡ The maximum dilution of each reagent was expressed for the direct binding ability to O-81 or NE-1 clones as determined in IF.

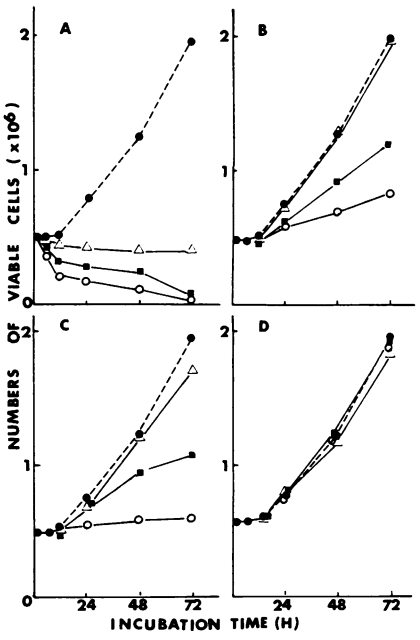

Figure 1. Different cytocydal effect of anti-Id-NCS conjugates on anti-DNA antibody-producing clones. Each cell suspension was first incubated with 1F5-NCS (*A*), D1E2-NCS (*B*), mouse Ig-NCS (*C*), or 1F5 alone (*D*) at the following doses of NCS or anti-Id: ●, 0 μg/ml; △, 0.04 μg/ml; ■, 0.2 μg/ml; and O, 1.0 μg/ml. After washing three times with media, the cells were again incubated and the viable cells counted at the indicated time as described in Methods.

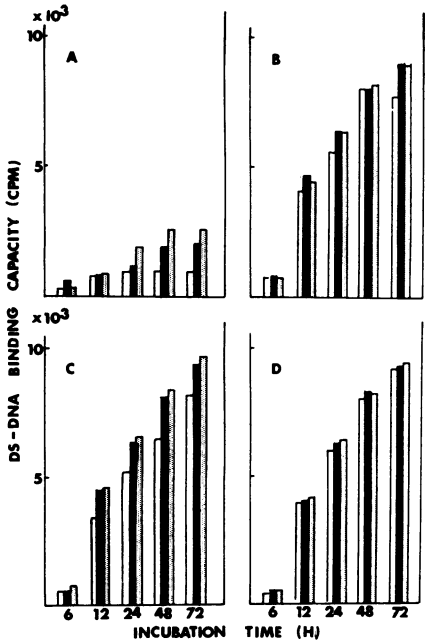

Figure 2. Suppressive effect of anti-Id–NCS to anti-DNA antibody production by the clones. The DNA-binding capacity was determined in the culture supernatant from each of the cells; they were treated with 1F5-NCS (*A*), DiE2-NCS (*B*), mouse Ig-NCS (*C*), or 1F5 alone (*D*) at the following doses of NCS or anti-Id: □, 0.04 μg/ml; ▣, 0.2 μg/ml; and ⊡, 1.0 μg/ml. The protocol for the experiments is the same as that in Fig. 1.

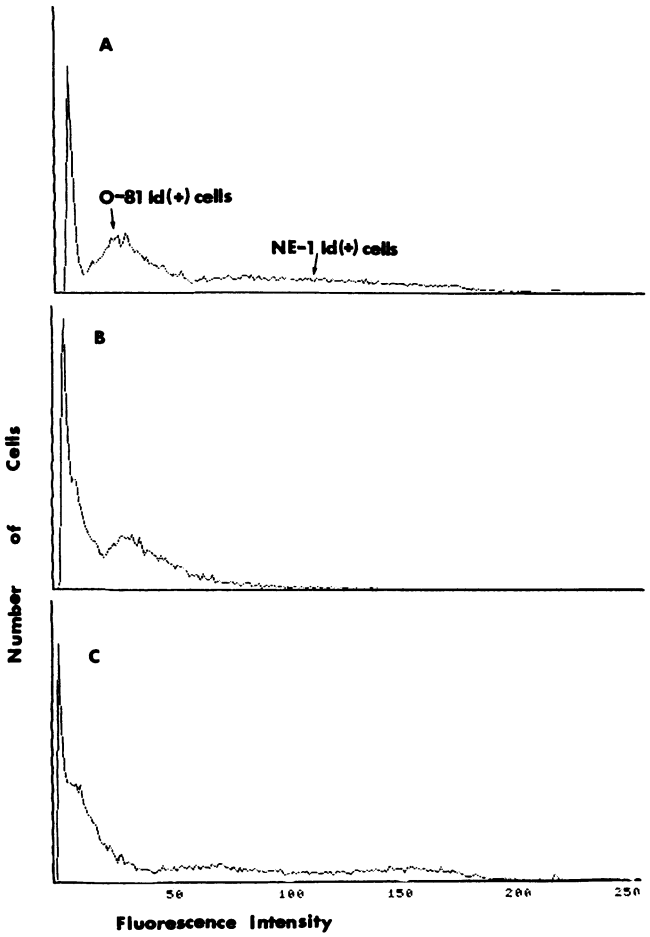

Figure 3. Fluorescence profiles of surface Id in mixture culture after the treatment of anti-Id–NCS. The mixture of O-81 and NE-1 cells was incubated with anti-mouse Ig-NCS (*A*), 1F5-NCS (*B*), or D1E2-NCS (*C*) at 0.05 μg/ml of NCS at 37°C for 6 h. After removing the conjugates by washing, each cell suspension was again incubated for 24 h at 37°C and then stained by D1E2 and 1F5, followed by fluorescein isothiocyanate–anti-mouse Ig $F(ab')_2$. *B* shows the elimination of NE-1–Id-positive cells after the treatment of 1F5-NCS, whereas specific killing of O-81–Id-positive cells was demonstrated in *C*.

demonstrates that only 1F5-NCS inhibited the production of anti-DNA antibodies.

The above results suggest a selective killing of autoantibody-producing clones by anti-Id antibody-conjugated NCS. To confirm this, D1E2-NCS was incubated with O-81, NE-1, NE-13, NE-28, NE-29, or SA-1 (EBV-transformed B cell clones not producing anti-DNA antibody) for 6 h at 37°C. After removing D1E2-NCS by washing three times with medium, each cell suspension was again incubated and tested for cell proliferation. Only O-81 clones failed to grow and to produce anti-DNA antibodies (data not shown). Similar results were obtained in a mixed culture consisting of O-81 and NE-1 clones with 1F5-NCS or D1E2-NCS. Fig. 3 shows the flow cytometry pattern for the idiotype expression in the latter, where the specific elimination of the clones by the relevant anti-Id–NCS was clearly demonstrated.

Discussion

This paper presents a new way for the specific elimination of autoantibody-producing clones. Would anti-Id antibodies be useful in the treatment of autoimmune states? Autoantibodies are a heterogenous population and bind to a large variety of antigens (17–20), suggesting a large number of different autoimmune clones. If this is true, eliminating a single Id may not affect the majority of pathogenetic autoantibodies, which bear Id that do not crossreact (9, 10). It has been demonstrated, however, that a limited number of anti-DNA antibodies, such as those with complement-fixing ability, cause the lesions seen in SLE (20, 21). It would, therefore, be necessary to only control a subpopulation of antibodies to DNA to protect against damage. In addition, studies have shown that the Id repertoire of anti-DNA autoantibodies might be rather restricted in autoimmune mice (22, 23). A high degree of Id crossreactivity was also found among human monoclonal anti-DNA antibodies (24) and the majority of sera in SLE (25). The anti-Id antibodies discussed in this paper, D1E2 and 1F5, also have the ability to react with widely shared Id of human anti-DNA antibodies from different patients with SLE (data not shown). Furthermore, the restricted anti-DNA antibody Id have been demonstrated in the glomerulus of SLE (26). These findings indicate that the use of mixed anti-Id reagents to dominant Id of anti-DNA autoantibodies responsible for tissue injury may be valuable for therapy (8). In this study, anti-Id antibodies specifically reacted with the corresponding Id-bearing clones on their surface, but this binding was not sufficient for an appreciable elimination of the clones, indicating that cross-linking of anti-Id antibody on the B cell surface did not inhibit cell proliferation. Other reports also demonstrate the inadequacy of therapy using anti-Id antibody alone. The suppressive effect of anti-Id antibody is only transient in an already established

antibody-producing autoimmune mice (9, 10). These observations prompted us to develop a new method for the manipulation of autoantibody production. We intended to abolish the anti-DNA antibody-producing clones by anti-Id antibody in combination with cytotoxic agents. This concept is based on the specific selection of anti-DNA clones by anti-Id antibodies followed by the cytocidal effect of NCS. Various types of drugs have been studied for their suitability in drug-antibody complexes. Methotrexate, daunomycin, and chlorambucil (27) have all been considered for use with antibody, and diphtheria toxin and ricin have been studied as a component of possible immunotoxins for tumor therapy (28, 29). These agents, however, have some disadvantages for clinical use because they sometimes cause nonspecific tissue injury (30). Our choice of NCS was based on the following considerations: (*a*) NCS is cytotoxic to lymphocytes as well as tumor cells (31); (*b*) NCS chromophore is more effective on a molar basis than other intercalating and DNA-degrading drugs (32); (*c*) Chemical alterations of apoprotein do not influence the active principle (chromophore); (*d*) There is no opening of covalent bonds to release cytostatic activity at the target; and (*e*) It is easily crosslinked with monoclonal antibody because of its solubility in water. We wanted to produce anti-Id conjugates while minimally denaturing the antibodies. We did this by using SPDP as a coupling agent, and it resulted in no decrease in either anti-Id binding or pharmacological activity of the bound NCS. The anti-Id–NCS conjugate was able to selectively eliminate the subpopulation of B cell clones bearing Id on their surface. In this study, EBV-immortalized cells were used as targets for anti-Id–NCS. These clones produce monoclonal anti-DNA autoantibody and represent a low to intermediate percentage of the population of activated B cells when fractionated on a discontinuous Percoll gradient. The cells also express Ia and B4, and weakly express B1 and B2 antigents on their surface. In addition, their Ig receptors share an Id with antigen receptors. These findings are identical with activated B cells, especially those found in fractions containing anti-DNA antibody-producing cells in peripheral blood lymphocytes of SLE (Takai, unpublished data). Therefore, these clones appear to serve as excellent models for anti-DNA autoantibody-producing cells in humans and for therapy with anti-DNA antibody production. We are currently asking if this method is valuable for altering anti-DNA production by human lymphocytes. This approach will also help to clarify the importance of specific clones in immune network regulation and may provide important clues about the pathogenesis of SLE.

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