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Effect of Borohydride Reduction on Antibodies

LIN PENG, GARY J. CALTON, AND JOSEPH W. BURNETT*

Division of Dermatology, Department of Medicine, University of Maryland School of Medicine, 22 South Greene Street, Baltimore, MD 21201

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ABSTRACT

The effect of borohydride reducing reagents on monoclonal and polyclonal antibodies was examined by enzyme-linked immunosorbent assay (ELISA). Each antibody showed different stability characteristics to the reducing reagents. Sodium cyanoborohydride was at least five times milder toward immunological activity than sodium borohydride, however, sodium cyanoborohydride with a catalytic amount of metal ion (Zn^{2+} or Al^{3+}) can be as harsh as sodium borohydride. Activated hydrophobic borohydrides, 9BBN-pyridine, did not have any advantages in respect to the stabilities of antibodies. Antibodies to be used for immunosorbent purification must be evaluated individually to determine whether their structure is stable to immobilization reagents and conditions prior to their linkage to the column support.

Index Entries: Borohydride reduction, effects of on antibodies; monoclonal and polyclonal antibodies; enzyme-linked immunosorbent assay (ELISA); sodium borohydride; sodium cyanoborohydride; 9BBN-pyridine; immunosorbent.

INTRODUCTION

Immunochromatography using monoclonal antibodies (Mab) immobilized on an insoluble matrix has been utilized with increasing frequency to purify proteins (*1*) because of the high degree of protein purity

*Author to whom all correspondence and reprint requests should be addressed.

obtainable in a single step (*2,3*). The most common method used for antibody immobilization utilizes cyanogen-bromide-activated Sepharose 4B (*4*). This activation procedure, however, has the disadvantage that labile isourea bonds are formed between the antibody and the matrix (*5,6*).

A stable immunosorbent system can be obtained by immobilization of antibody via an oxidation–reduction procedure, producing an amine linkage. This procedure involves formation of a Schiff base between a carbonyl group and an amine followed by reduction of a Schiff base with a reducing agent, such as sodium borohydride. Investigators who have used this technique have experienced difficulty in achieving immobilization with a constantly high retention of immunological activity (*7–10*). No explanation of this difficulty has been given, but two areas of investigation, the formation of the Schiff base and the reduction by metal hydride, are possible chemical steps that could well affect the degree of antibody binding. The effect of various borohydride reducing agents, such as $NaBH_4$, $NaCNBH_3$, and 9-borobicyclo[3,3,1]nonane-pyridine (9BBN-pyridine) on different Mab available in our laboratories was evaluated in order to define conditions that were sufficiently mild for immobilization of Mab in a stable conformation in reasonable yield.

MATERIALS AND METHODS

Bacillus anthracis protective antigen (PA), lethal factor (LF), mouse anti-PA Mab, mouse anti-LF Mab, and mouse anti-PA polyclonal antibody were provided by USARIID, Fort Detrick, MD. Jellyfish (*Chrysaora quinquecirrha*) venom and a protective anti-*Chrysaora* Mab were prepared in our lab as previously described (*11*). Goat anti-human IgG (GaHGG) was a gift of Purification Engineering, Inc., Columbia, MD. Human IgG was prepared from pooled serum by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography. The $NaCNBH_3$ and 9BBN-pyridine were purchased from Aldrich Chemical Co., Milwaukee, WI; $NaBH_4$, $ZnSO_4$, and $AlCl_3$ were reagent grade from J. T. Baker Co., Phillipsburg, NJ. Isotype reagent rabbit anti-mouse IgG was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN; alkaline phosphatase rabbit anti-mouse IgG and rabbit anti-goat IgG were purchased from Miles Laboratory, Inc., Naperville, IL; *p*-nitrophenylphosphate (Sigma 104) was purchased from Sigma Chemical Co., St. Louis, MO.

Reductions

Antibody (10 μg) and reducing reagents were incubated in 1.0 mL of 0.1*M* ethanolamine, 0.01*M* phosphate buffer, pH 7.6, at 25°C for 4 h.

Enzyme-Linked Immunosorbent Assay

Antigen was coated on 96-well plates (*3,11*), and antibody (100 μL) was added to each well. After incubation at 37°C for 1.5 h, the plates

were washed with PBS-Tween, pH 7, buffer four times. Rabbit anti-mouse enzyme conjugate or rabbit anti-goat enzyme conjugate was added and incubated at 37°C for 1 h, after which the plate was washed again with PBS-Tween four times. The *p*-nitrophenylphosphate in diethanolamine buffer (100 μL) was added and the mixture incubated at 37°C for 15 min. Sodium hydroxide (3*M*, 50 μL/well) was then added to stop the reaction. The enzyme-linked immunosorbent assay (ELISA) plate was read at 405 nm. The data obtained had a variance of less then 7%.

Rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, and control serum were used to subtype the mouse anti-LF, anti-PA, and anti-*Chrysaora* Mabs. All three Mabs were in subclass IgG1.

RESULTS

The stability of goat anti-human IgG, mouse anti-PA (polyclonal and monoclonal), and anti-LF (10 μg each) were examined under sodium borohydride ($NaBH_4$) reduction conditions (Fig. 1). The immunological activity of three antibodies decreased with exposure to sodium borohydride. Polyclonal mouse anti-PA activity was more stable at concentrations above 10 μmol after contact with this reducing agent than with the Mab.

Mouse anti-PA (50 μg) incubated with 100 μmol of sodium borohydride at 25°C was used to determine the effect of time on the degradation of immunological activity of the antibody. The activity of the antibody decreased 10% in 20 min and approximately 40% in 3 h under these conditions.

Mouse anti-LF and anti-PA Mab were stable in the presence of 50 μmol sodium cyanoborohydride. Both decreased in activity at higher levels of sodium cyanoborohydride (Fig. 2). In contrast, mouse anti-*Chrysaora* Mab was unaffected by $NaCNBH_3$ at concentrations up to 500 μmol (Fig. 2). Goat anti-human IgG activity was very stable toward sodium cyanoborohydride since no decrease in the activity of the antibody was observed even at 1250 μmol $NaCNBH_3$.

When a catalytic amount of zinc sulfate or aluminum chloride was used with $NaCNBH_3$ the immunological activity of both mouse anti-LF and anti-PA Mab was decreased substantially (Fig. 3).

Sterically hindered reducing agents, such as 9BBN-pyridine, had no effect on the reactivity of goat anti-human IgG or anti-*Chrysaora* Mab, but degraded the activity of anti-PA Mab at the 10-μmol and anti-LF at the 20-μmol levels (Fig. 4).

DISCUSSION

Studies on the stability of antibodies under borohydride reduction conditions showed that each antibody responded differently (Figs.

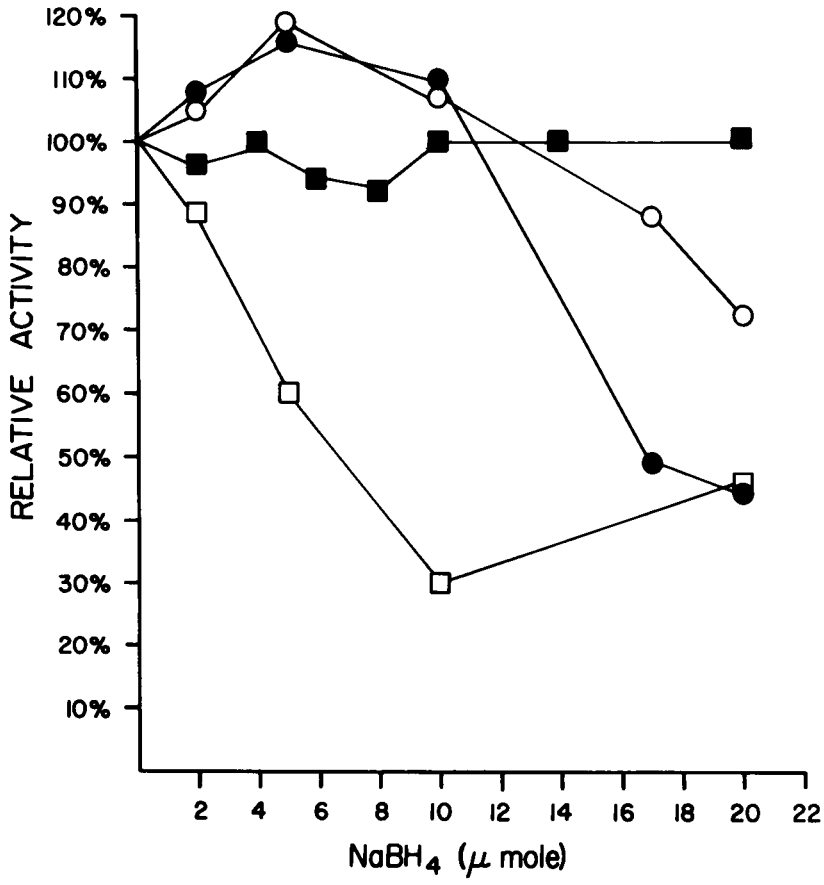

Fig. 1. Effect of sodium borohydride on antibodies. Antibodies (10 μg) were incubated with sodium borohydride ($NaBH_4$) for 4 h: Goat anti-human IgG (O); mouse anti-PA Mab (●); mouse anti-PA polyclonal (■); and mouse anti-LF Mab (□).

1,2,4). Polyclonal goat immunoglobulins were quite stable as compared to the Mabs. There was variation in Mab stability to borohydride reduction. Mouse anti-PA was more labile in the presence of $NaCNBH_3$, whereas anti-LF was more sensitive to $NaBH_4$ (Figs. 1 and 2). The activity of both anti-LF and anti-PA was destroyed by 9BBN-pyridine, but anti-*Chrysaora* activity remained intact in the presence of a relatively high concentration of the same reagent (Fig. 4).

Two mouse Mabs were tested with $NaBH_4$ (Fig. 1); one (anti-LF) lost reactivity at a concentration of 0.2 μmol/μg, the other (anti-PA), as well as GaHGG, began to lose its activity at about 1.0 μmol $NaBH_4$/μg antibody. The $NaCNBH_3$ was a milder reducing reagent than $NaBH_4$, a fact that allows higher concentrations of $NaCNBH_3$ to be placed in contact with the antibodies (Figs. 1 and 2). At least 5-fold higher concentrations of $NaCNBH_3$ as compared to $NaBH_4$ could be used to treat antibodies without damaging antibody activity. Catalytic amounts of metal ions, such as zinc (Zn^{2+}) or aluminum (Al^{3+}), are commonly used with

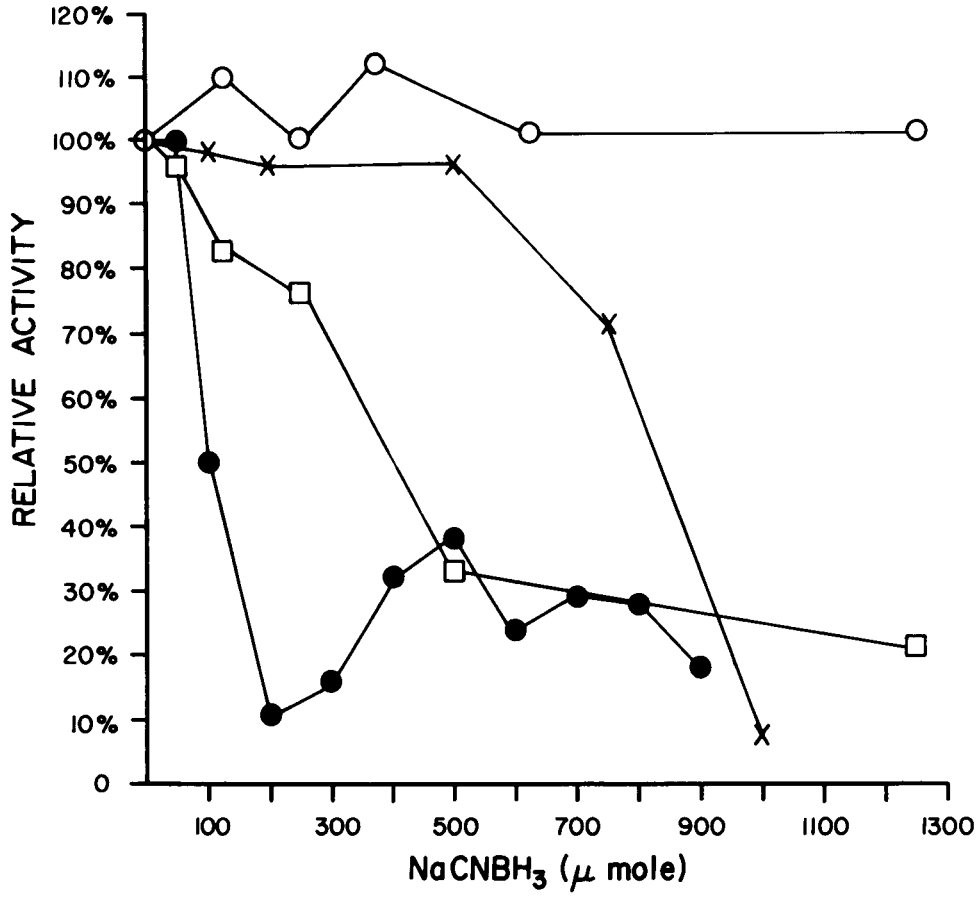

Fig. 2. Effect of sodium cyanoborohydride on antibodies. Antibodies (10 μg) were incubated with sodium cyanoborohydride ($NaCNBH_3$) for 4 h: Goat anti-human IgG (O); mouse anti-PA Mab (●); mouse anti-LF Mab (□); and mouse anti-*Chrysaora* Mab(X).

$NaCNBH_3$ in order to lower the concentration of cyanide ion (CN^-), thus reducing undesired side reactions and increasing the reaction rate (*12*). However, these metal ions in conjunction with $NaCNBH_3$ resulted in significant reduction of antibody activity even when used at low concentrations (Fig. 3).

Loss of immunological activity of an antibody may be caused by either chemical or conformational alteration of the antibody. The $NaBH_4$ is known to cause degradation of proteins by the reduction of disulfide bonds. Lack of stability of Mab toward borohydride was not related to the origin of the antibodies since the polyclonal mouse anti-PA was more stable than its monoclonal counterpart in $NaBH_4$, and mouse anti-*Chrysaora* Mab was more stable than mouse anti-PA or anti-LF Mab in either $NaCNBH_3$ or 9BBN-pyridine. The most vulnerable structure in proteins with respect to reducing reagent is the disulfide bond. Disulfide bonds in enzymes were generally stable under moderate concentration of

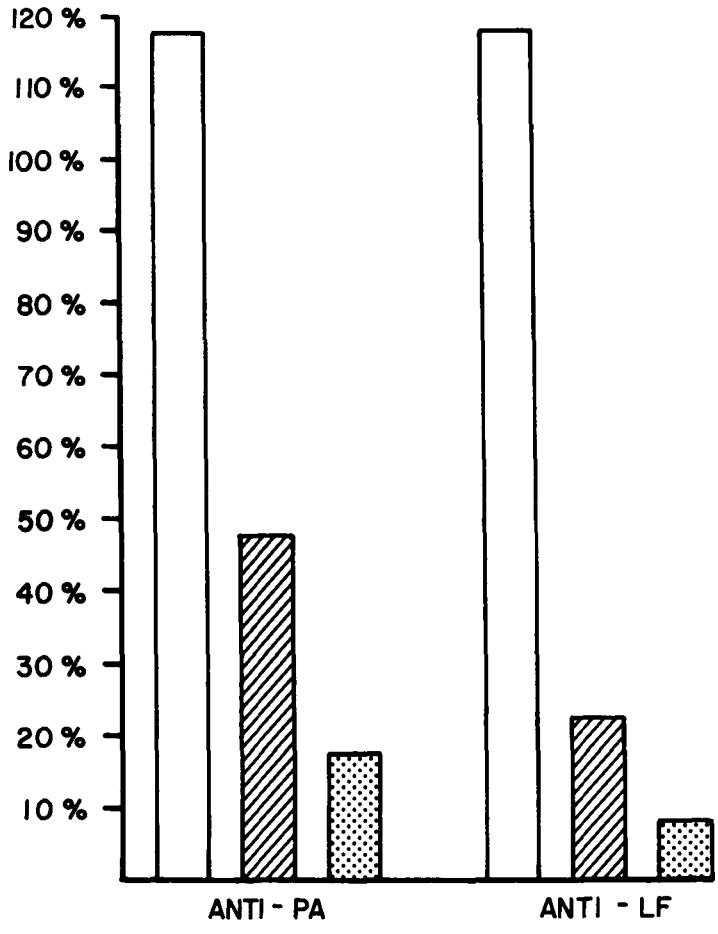

Fig. 3. Effect of sodium cyanoborohydride on antibodies in the presence of catalytic amount of metal ions. Antibodies (100 μg) were incubated with sodium cyanoborohydride (80 μmol) (□) and with either zinc sulfate (4 μmol) (▤) or aluminum chloride (5 μmol) (▦).

$NaBH_4$ (10 m*M*/mg) or high concentration of $NaCNBH_3$ (80 m*M*/mg) (*13*). The stability of disulfide bonds in proteins is partially protected because of the folding of the molecule. High concentrations of urea (8*M*) were required to unfold proteins before disulfide bonds could be reached by hydride ions (*14*). The antibodies used in this investigation were more vulnerable to hydride compounds than protein enzymes used in earlier studies, which suggests that antibody disulfide bonds may be more readily attacked by hydride ions.

In these investigations, ELISA was used as an indicator of the effect of borohydride reducing agents on antibodies. It is to be emphasized that antibody immobilized on Sepharose 4B or cellulose beads are only 10–20% active, whereas antibody coated to an ELISA plastic plate is assumed to be fully active. Thus, a reduction of antibody activity caused by

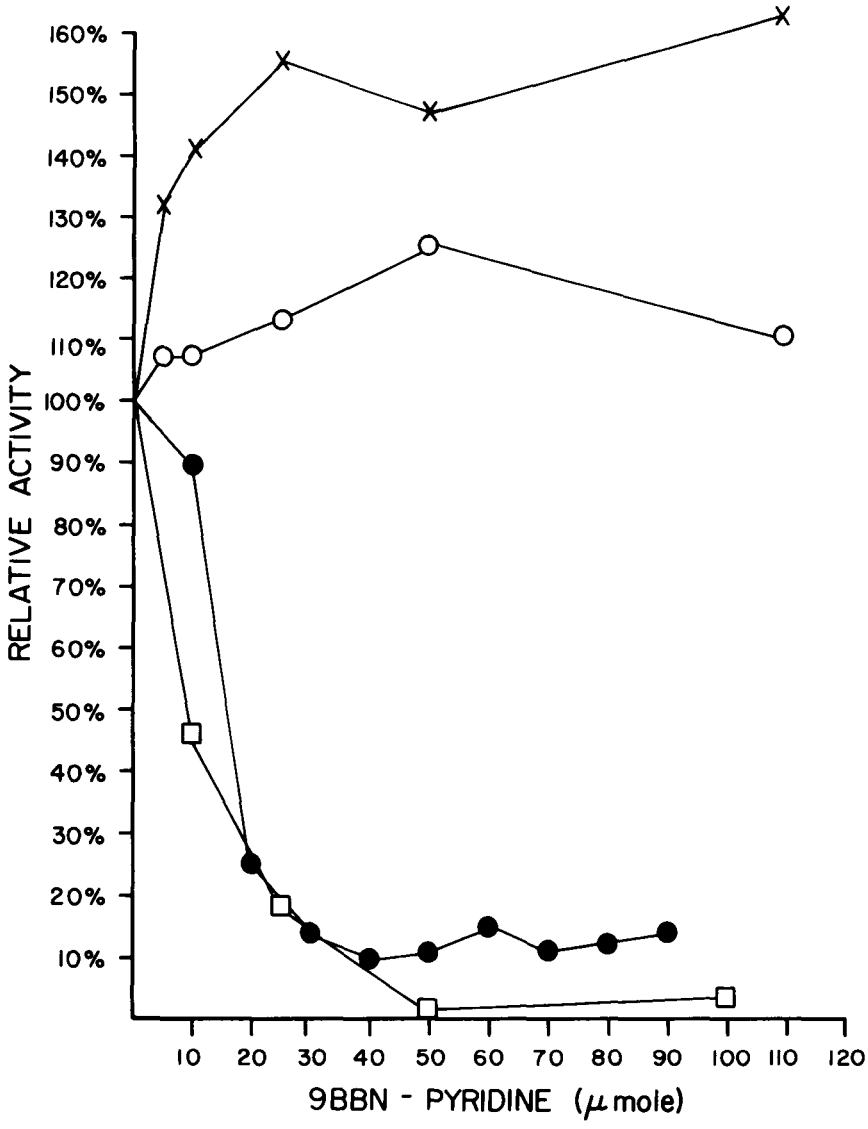

Fig. 4. Effect of 9BBN-pyridine on antibodies. Antibodies (10 μg) were incubated with 9BBN-pyridine for 4 h: Goat anti-human IgG (O); mouse anti-PA Mab (•); mouse anti-LF Mab (□); mouse anti-*Chrysaora* Mab (X).

borohydride treatment on the ELISA plate may be more pronounced if the antibody had been immobilized in a column support.

Immunosorbent purifications require stable linkages to prevent contamination of the purified protein with antibody leaking from the support media. In addition, the lack of appropriate immobilization conditions for the selected antibodies can result in low yields of activity by the immunosorbent (*7,8*). The evaluation of Mab for use in immunochromatography has been discussed previously (*15*). This study indicates that

Mabs selected for immobilization must also be characterized for stability to the immobilization reagents and conditions in order to obtain high yields of activity upon immobilization. Sufficiently mild conditions may be obtained to provide excellent immobilization yields with stable linkages if this evaluation is carried out.

SUMMARY

The effect of sodium borohydride, sodium cyanoborohydride, and 9BBN-pyridine reducing treatment on antibodies was examined by the ELISA method. Each antibody studied showed different chemical stability to reducing reagents.

Sodium cyanoborohydride was less deleterious against antibody immunological activity than sodium borohydride unless a catalytic amount of metal ion (Zn^{2+} or Al^{3+}) was also used. Activated hydrophobic borohydride did not offer an advantage in stabilizing the antibody. It is to be stressed that antibodies selected for immobilization must be characterized and studied to ensure that optimal conditions for binding to immunosorbent supports are known.

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