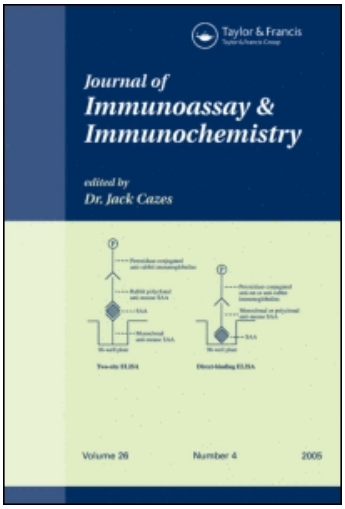

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Enzyme-Linked Immunosorbent Assay of Neocarzinostatin Chromophore (NCS-CHR) by Use of a Monoclonal Antibody Against NCS-CHR Analog

Michinao Mizugaki[a]; Kunihiko Itoh[a]; Hironori Nakamura[a]; Nakao Ishida[b]
[a] Department of Pharmaceutical Sciences, Tohoku University Hospital, Sendai [b] The Sendai Institute of Microbiology, Sendai, Japan

To cite this Article Mizugaki, Michinao , Itoh, Kunihiko , Nakamura, Hironori and Ishida, Nakao(1996) 'Enzyme-Linked Immunosorbent Assay of Neocarzinostatin Chromophore (NCS-CHR) by Use of a Monoclonal Antibody Against NCS-CHR Analog', Journal of Immunoassay and Immunochemistry, 17: 2, 133 — 144
To link to this Article: DOI: 10.1080/01971529608005784
URL: http://dx.doi.org/10.1080/01971529608005784

JOURNAL OF IMMUNOASSAY, 17(2), 133-144 (1996)

ENZYME-LINKED IMMUNOSORBENT ASSAY OF NEOCARZINOSTATIN CHROMOPHORE (NCS-CHR) BY USE OF A MONOCLONAL ANTIBODY AGAINST NCS-CHR ANALOG

Michinao Mizugaki[1], Kunihiko Itoh[1], Hironori Nakamura[1], and Nakao Ishida[2]
[1]Department of Pharmaceutical Sciences, Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku Sendai 980-77, [2]The Sendai Institute of Microbiology, 2-5-22 Ichibancho, Aoba-ku, Sendai 980, Japan

ABSTRACT

A monoclonal antibody against NCS-chr was prepared and characterized. Because of the instability of NCS-chr, chemically synthesized stable analog compound, termed PS, was used as a hapten of immunogen. The obtained antibody, termed APS, reacted with NCS-chr, but neither with NCS, NCS-polystyrene-maleic acid conjugate (SMANCS), nor UV-irradiated NCS-chr. Epitope analysis using the compounds that have a structure similar to PS showed that APS recognized the total structure, particularly cyclopenten moiety, of PS. These results suggest that APS recognizes the enediyne structure of NCS-chr. Next, the inhibition enzyme-linked immunosorbent assay (ELISA) for determination of NCS-chr was established. The standard curve showed that the μg order of NCS-chr were accurately measurable by the established ELISA. Furthermore, it was revealed that the established ELISA was more sensitive than the antibiotic activity determination, termed Bio-assay. The established ELISA will be useful as a quantitative method of NCS-chr.

(KEY WORDS: Neocarzinostatin-chromophore, monoclonal antibody, enzyme-linked immunosorbent assay)

INTRODUCTION

Neocarzinostatin (NCS), a natural chromoprotein antibiotic isolated from *Streptomyces carzinostatics*, is composed of a 113-amino acid protein component

(apo-NCS) and a labile, nonprotein chromophore component (NCS-chr) (1,2). A potent cytotoxic agent, NCS has undergone clinical evaluation for antitumor activity (3,4). In the presence of a thiol cofactor, NCS induces cleavage of single- and double-stranded DNA both *in vivo* and *in vitro*. The cleavage activity resides exclusively within the chromophore component (5,6), whose structure (7) was shown previously to include the epoxybicyclo[7.3.0]dodeca-dienediyne structural element. *In vitro*, NCS-chr undergoes efficient thiol addition to form a highly reactive, carbon-centered biradical, which provides a potential molecular basis for the antitumor and DNA-cleaving properties of NCS (8). Studies on the mode of action of NCS-chr using NCS-chr analogs have also been reported (9-11). From these observations, direct determination of the actual amount of NCS-chr is necessary to evaluate the antitumor activity of NCS as well as a NCS-polystyrene-maleic acid conjugate (SMANCS).

The amount of NCS, SMANCS or NCS-chr has been measured with an antibacterial activity against *Micrococcus luteus* (*M. luteus*), termed Bio-assay (12), but this technique is laborious, unreliable, and time-consuming. The monoclonal antibody-based immunoassay system is considered to be the most suitable method to determine NCS-chr in view of the specificity, sensitivity, rapidity, and simplicity. Because NCS-chr has an extremely unstable structure, a stable NCS-chr analog, termed PS, chemically synthesized by Hirama *et al.* (9,10) (Fig. 1) was used as a hapten, and monoclonal antibody producing-hybridoma cells were prepared by using a PS-conjugate. Finally, we obtained a monoclonal antibody reactive with natural NCS-chr.

In this paper, we describe the preparation and characterization of a monoclonal antibody against NCS-chr, and the establishment of an enzyme-linked immunosorbent assay (ELISA) for NCS-chr.

MATERIALS AND METHODS

Preparation of immunogen

The PS (0.1 mmol) in pyridine was reacted with succinic anhydride (0.2 mmol), and stirred for 24 hr at room temperature. After the pyridine was evaporated

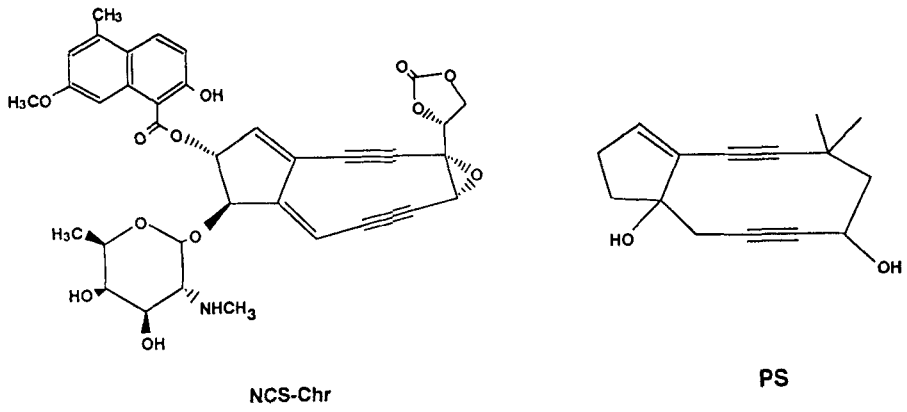

FIGURE 1 Chemical structure of NCS-chr analog used to prepare a monoclonal antibody against NCS-chr.

in vaccuo, the reaction mixture was extracted with chloroform under an acidic condition. The organic layer was dried over anhydrous sodium sulfonate and evaporated *in vaccuo*.

The obtained PS-succinic half ester derivative (18 μmol) was dissolved with 70% dioxane in water, next N-hydroxy succinimide (37 μmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (36 μmol) was added, then stirred for 2 hr at room temperature. The reaction mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfonate and evaporated *in vaccuo*. The resulting product was dissolved with 50 μl of dimethyl sulfoxide, and added dropwise to a solution of bovine serum albumin (BSA) in pH 7.4 phosphate-buffered saline (PBS) (10 mg/ml). After overnight incubation at 4℃, the hapten-carrier protein conjugate was dialyzed against PBS. The lyophilized product was stored at -20℃ until use.

Immunization

Female BALB/c mice (6 weeks old) were immunized *i.p.* and *s.c.* with the PS-BSA conjugate (100 μg/mouse) in Freund's complete adjuvant. The second and

third immunization were done at 14-day intervals by an *i.p.* injection of the PS-BSA conjugate in Freund's incomplete adjuvant. Ten days after the third immunization, the mouse with the highest antibody titer received a booster *i.v.* injection with 80 μg/mouse of PS-BSA in 0.2 ml of PBS and was sacrificed three days later.

Preparation of hybridoma

Spleen cells of the mouse were fused with Sp2/0-Ag14-K13 mouse myeloma cells in the presence of 50% (w/v) polyethylene glycol 4000 [13]. Fused spleen-myeloma cells were selected by culturing in HAT medium (RPMI medium containing 2 mM glutamine, 0.2% glucose, 1 mM pyruvic acid, penicillin at 100 U/ml, streptomycin at 100 μg/ml and 10% heat-inactivated fetal calf serum (FCS) (Standard medium) supplemented with 100 μM hypoxantine, 0.4 μM aminopterin and 16 μM thymidime). Cultures were maintained in a 5% CO_2 incubator at 37℃. When hybridoma colonies appeared, they were expanded and maintained in HT medium (HAT medium without aminopterin) and finally in the standard medium.

The supernatants of hybridoma cell cultures were screened by direct ELISA for production of anti-PS antibodies. The PS-human serum albumin (HSA) conjugate was used as an antigen to exclude the antibodies reactive with BSA. Then selected hybridomas were cloned by a limiting dilution method using mouse thymocytes as feeder cells.

Inhibition ELISA

A 100 μl aliquot of the PS-HSA conjugate dissolved in PBS (10 μg protein/ml) was fixed to the polyvinyl chloride microtiter wells (MS-7196 F, Sumitomo Bakelite, Tokyo, Japan) by overnight incubation at 4℃. The wells were then filled with 100 μl of 3% skim milk in PBS. After 1 hr incubation at 37℃, the solution was discarded and 50 μl of serially diluted authentic NCS-chr solutions or NCS-chr related compounds were added followed by the addition of an equal volume of antibody solution (10 μg/ml) and incubated for 1 hr at 4℃ in the dark. After the wells were washed 3 times with 0.05% Tween 20-PBS and twice with

PBS, the wells were filled with 100 μl of 1:3000 diluted alkaline phosphatase (ALP) labeled goat anti-mouse IgG (final conc. 1.7 μg/ml) (Tago, Burlingame, CA, USA) followed by incubation for 45 min at 4℃. After the same washing procedure as above, 100 μl of *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate) dissolved in 1 M diethanolamine buffer (pH 9.8) (1 mg/ml) was added and incubated for 30 min at 37℃. The color development was stopped by addition of 2 M sodium hydroxide, and the absorbance of the resultant *p*-nitrophenol in each well was measured at 405 nm by a microplate reader (UV max, Molecular Devices, Sunnyvale, CA, USA).

Bio-assay

Anti-bacterial activity of NCS-chr was determined by the conventional paper disk diffusion method [11]. *M. luteus* stored at -80℃ was thawed and cultured overnight with meat extract bouillon at 37℃. The bacterial culture was mixed into an agar-polypepton solution, then poured into a rectangle shaped plastic dish. Paper disks soaked in various concentration of NCS-chr solution (20 μl) were put on the agar plate, and incubated for 48 hr at 37℃ in the dark. The estimation of bio-activity of NCS-chr was determined by the diameter of bacterial growth-inhibition circle on the agar.

RESULTS

Characterization of APS monoclonal antibody

Anti-NCS-chr antibody-producing hybridomas were identified by screening for NCS-chr inhibition of hybridoma supernatants binding to PS-HSA using the inhibition ELISA. Consequently, seven monoclonal antibodies reactive with NCS-Chr were obtained. Among these antibodies, 1-3-19, termed APS (IgG_1, κ) was selected because it had the highest reactivity against NCS-chr in the inhibition ELISA. The reactivity of APS against NCS-chr related compounds was determined using the inhibition ELISA. APS reacted with NCS-chr, but not with NCS, SMANCS, and non-bioactive NCS-chr resulting from UV-irradiation (Table 1). It was revealed that APS reacts with only NCS-chr that retains its biological activity.

TABLE 1

Reactivity of APS monoclonal antibody against NCS-chr and its related compounds

Inhibitor	IC_{50} (mg/ml)
NCS-chr	1.2
NCS	>200
SMANCS	>200
UV-NCS-chr	>200

Epitope analysis of APS monoclonal antibody

By using compounds that have a structure similar to PS (Fig. 2), the epitope of APS was determined. APS reacted with a ten-membered ring compound [2] as strongly as it did with PS [1], but did not react with the compound [3] that had an open structure of the ten-membered ring of PS. APS had no reactivity against the compounds that had a hydroxyl group [4] or a large substituent group [5] in a five-membered ring even though they had the same ten-membered ring structure as PS [1] and compound [2]. These results suggest that APS predominantly recognizes the cyclopentene moiety of PS with ten-membered ring structure.

Establishment of inhibition ELISA for determination of NCS-chr

Using APS monoclonal antibody, we established an inhibition ELISA to quantitate NCS-chr. The concentration of PS-HSA and APS was optimized by antigen-antibody dual titration. A standard curve is shown in Fig. 3. NCS-chr solutions from 2 mg/ml to 75 μg/ml were measurable by the ELISA, and the detection limit was calculated to be 2.5 μg from the standard curve. The intra- and inter-assay variations were within 10% (data not shown).

Comparison of established ELISA and Bio-assay

Next, the established ELISA was compared to the Bio-assay. The serially diluted NCS-chr (31-1000 μg/ml) was divided into the two parts, then analyzed by

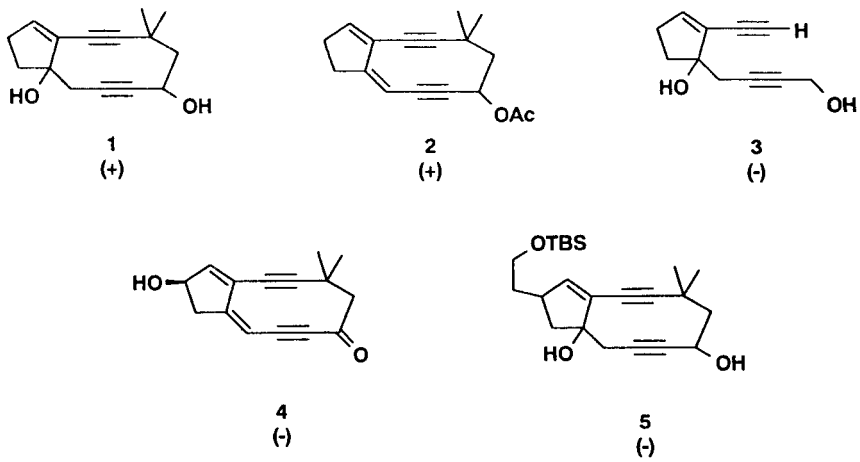

FIGURE 2 Chemical structure of compounds used for epitope analysis of APS monoclonal antibody.

The reactivity of APS to compounds are shown in the parentheses. (+); strongly reactive, (-); non reactive.

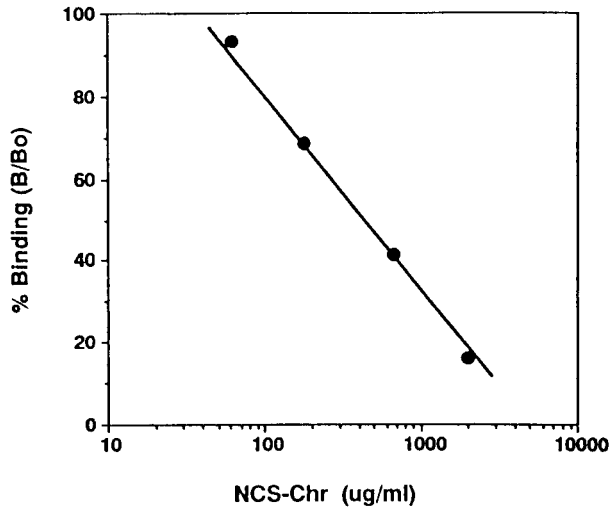

FIGURE 3 Standard curve for determination of NCS-chr.

Data are given in the form of a log-logit plot. Points: mean values of triplicate determinations.

the inhibition ELISA and the Bio-assay, respectively. As shown in Fig. 4, a good correlation was observed between these assays (r=0.9958). Furthermore, the established ELISA appeared to be more sensitive than the Bio-assay. Although 30 μg/ml of NCS-chr was accurately determined by the inhibition ELISA (around 50% inhibition), the same concentration of NCS-chr was beyond the detection limit of the Bio-assay (Fig. 4). With regard to sensitivity, rapidity, and simplicity, the established ELISA would be more useful than the Bio-assay for determination of NCS-chr.

DISCUSSION

NCS and its derivative, SMANCS have been widely used for cancer chemotherapy (3,4). The anti-tumor effect of NCS has been attributed to its chromophore which has a characteristic enediyne structure. For the exact clinical evaluation of NCS, the actual amount of NCS-chr should be determined by another method other than the Bio-assay. Because we have studied the monoclonal antibody-based immunoassay for the compounds of a low molecular weight (14-17), we experimented by applying this methodology to the quantitation of NCS-chr. As it is well known that NCS-chr without apo-NCS degrades rapidly in aqueous solution (2), we used the chemically synthesized stable analog compound that has a structure closely similar to NCS-chr (Fig. 1). As a result, we obtained the monoclonal antibody reactive with NCS-chr. Selective reactivity of APS to NCS-chr (Table 1) and its proposed PS recognition site (Fig. 2) suggest that APS recognizes the enediyne structure of NCS-chr. It was proven that a specific monoclonal antibody could be obtained by using not only the compound of interest but also the analog compound as a hapten for immunization. This methodology might be applicable for making monoclonal antibodies against the naturally occurring compounds that have a known structure with unstable and are at low concentrations.

NCS-chr levels were successfully determined by the established ELISA using the APS monoclonal antibody. Furthermore, it was revealed that the established ELISA had a higher sensitivity than that of the Bio-assay (Fig. 4).

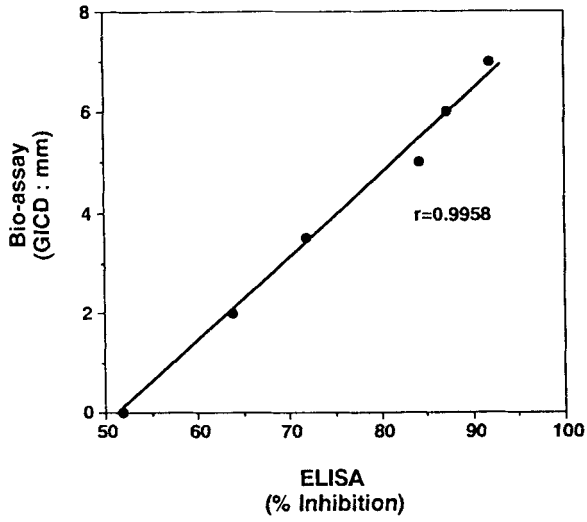

FIGURE 4 Comparison of the inhibition ELISA and the Bio-assay.
r: correlation coefficient, GICD: growth inhibition circle diameter.

However, the analytical sensitivity of the established ELISA was limited. The range of NCS-chr concentration that could be measured by the established ELISA was from μg/ml to mg/ml (Fig. 3). In general, the sensitivity of ELISA should be in the range of pg/ml to ng/ml. The cause of the low sensitivity may be a low affinity of APS for NCS-chr. NCS-chr has moieties of a naphthalene carboxylic acid and an amino sugar in the enediyne core structure, while PS does not have these groups (Fig. 1). These moieties may prevent the antibody-antigen interaction. Semisynthetic combinatorial antibody library technology makes it possible to increase the affinity of antibody to the antigen of interest (18) or to change the specificity of antibody (19,20). Using the APS monoclonal antibody as a starting material, a novel antibody that has a high affinity to NCS-chr might be successfully obtained with this technique.

In conclusion, we obtained a monoclonal antibody reactive with NCS-chr using a NCS-chr analog as a hapten for immunization, and established an ELISA with a sensitivity higher than that of the conventional Bio-assay. This assay system

will be useful for rapid determination of actual amount of NCS-chr in biological samples. Furthermore, APS monoclonal antibody itself might be applicable for screening of the novel enediyne compounds from the bacterial culture supernatants.

ACKNOWLEDGMENTS

We are very thankful to Professor Masahiro Hirama (Department of Chemistry, Tohoku University Faculty of Science) for providing chemically synthesized NCS-chr analog compounds. This study was supported in part by a Grant-in-Aid from the Sendai Institute of Microbiology and POLA Kasei.

REFERENCES

1. Ishida, N., Miyazaki, K., Kumagai, K. and Rikimaru, M. Neocarzinostatin, an antitumor antibiotic of high molecular weight; Isolation, physicochemical propaties and biological activities. J. Antibiotics 1965; Ser. A 18: 68-76.

2. Koide, Y., Ishii, F., Hasuda, K., *et al.* Isolation of a non-protein component and a protein component from neocarzinostatin (NCS) and thier biological activities. J. Antibiotics 1980; 33: 342-6.

3. Kimoto, A., Konno, T., Kawaguchi, T., Miyauchi, Y. and Maeda, H. Anti-tumor effect of SMANCS on rat mammary tumor induced by 7,12-dimethylbenz[a]anthracene. Cancer Res. 1992; 52: 1013-17.

4. Konno, T. Targeting chemotherapy for hepatoma: Arterial administration of anticancer drugs dissolved in lipiodol. Eur. J. Cancer 1992; 28: 403-9.

5. Kappen, L.S., Chen, C. and Goldberg, I.H. Atypical abasic sites generated by neocarzinistatin at sequence-specific cytidylate residues in oligodeoxynucleotides. Biochemistry 1988; 27: 4331-40.

6. Lee, S.H. and Goldberg, I.H. Sequence-specific, strandselective, and directional binding of neocarzinostatin chromophore to oligodeoxyribonucleotides. Biochemistry 1989; 28: 1019-26.

7. Edo,K., Mizugaki, M., Koide, Y., *et al.* The structure of neocarzinostatin chromophore possessing a novel bicyclo[7,3,0]dodecadiyne system. Tetrahedoron Lett. 1985; 26: 331-4.

8. Myers, A.G. and Proteau, P.J. Evidence for spontaneous, low-temperature biradical formation from a highly reactive neocarzinostatin chromophore-thiol conjugate. J. Am. Chem. Soc. 1989; 111: 1146-7.

9. Hirama, M., Fujiwara, K., Shigematu, K. and Fukuzawa, Y. The 10-membered ring analogues of neocarzinostatin chromophore: Design, synthesis, and mode of decomposition. J. Am. Chem. Soc. 1989; 111: 4120-2.

10. Fujiwara, K., Sakai, H. and Hirama, M. Enyne[3]cumulene. Synthesis and mode of aromatization. J. Org. Chem.1991; 56: 1688-9.

11. Doi, T. and Takahashi, T. Syntheses and transannulation cyclization of neocarzinostatin-chromophore and esperamicin-calichemicin analogues. J. Org. Chem. 1991; 56: 3465-7.

12. Neocarzinostatin, Minimum requirement of antibiotic products in Japan. 1986: 403-5.

13. Kohler, G. and Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975; 256: 495-7.

14. Itoh, K., Mizugaki, M. and Ishida, N. Preparation of a monoclonal antibody specific for 1-methyladenosine and its application for the detection of elevated levels of 1-methyladenosine in urines from cancer patients. Jpn. J. Cancer Res. (GANN) 1988; 79: 1130-8.

15. Itoh, K., Mizugaki, M. and Ishida, N. Detection of elevated amounts of urinary pseudouridine in cancer patients by use of a monoclonal antibody. Clin. Chim. Acta 1989; 181: 305-16.

16. Mizugaki, M., Itoh, K., Hayasaka, M., *et al.* Monoclonal antibody-based enzyme-linked immunosorbent assay for glycyrrizin and its aglycon, glycyrrhetic acid. J. Immunoassay 1994; 15: 21-34.

17. Itoh, K., Aida, S., Ishiwata, S., Yamaguchi, T., Ishida, N. and Mizugaki, M. Immunochemical detection of urinary 5-methyl-2'-deoxycytidine as a potent biologic marker for leukemia. Clin. Chim. Acta 1995; 234: 37-45.

18. Barbas, C.F.III, Hu, D., Dunlop, L., *et al.* In vitro evaluation of a neutralizing human antibody to human immunodeficiency virus type 1 to enhance affinity and broaden strain cross-reactivity. Proc. Natl. Acad. Sci. USA 1994; 91: 3809-13.

19. Barbas, C.F.III, Bain, J.D., Hoekstra, D.M., and Lerner, R.A. semisynthetic combinatorial antibody libraries: A chemical solution to the diversity problem. Proc. Natl. Acad. Sci. USA 1992; 89: 4457-61.

20. Barbas, C.F.III, Amberg, N., Simoncsits, A., Jones, T.M., and Lerner, R.A. Selection of human anti-hapten antibodies from semi-synthetic library. Gene 1993; 137: 57-62.