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CONSTRUCTION AND ANALYSIS
OF RECOMBINANT MOUSE ANTI-BOTULINUM ANTIBODIES

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Anti-botulinum neurotoxin antibodies were produced by cloning heavy and light chain genes from an immunized mouse. Selection by phage display against immobilized botulinum toxin subtype B allowed for the isolation of multiple high affinity Fab producing cell lines. These clones can be directed to express large quantities of Fab antibody at a significantly lower cost than hybridoma cell culture. Purification can be simplified by engineering an affinity tag directly into the nucleotide sequence of each antibody clone. Methods of construction and analysis are presented along with affinity constant determination and nucleotide sequencing data.

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CONSTRUCTION AND ANALYSIS OF RECOMBINANT MOUSE ANTI-BOTULINUM ANTIBODIES

1. INTRODUCTION

The probability of isolating antibodies to epitopes of an antigen which are not immunodominant is limited to a small portion of the immunoglobulins in the mouse repertoire.. The diagnostic and therapeutic uses of monoclonal antibodies often require high affinities that can be difficult to obtain with a standard fusion[1]. The need to isolate a larger proportion of the immune repertoire has prompted researchers to develop techniques for the construction of recombinant antibody libraries[2-7]. A cDNA library can be made from messenger RNA (mRNA) isolated from the spleens of immunized mice. The cloned mouse heavy and light chain genes can be expressed in *E. coli* to produce single chain or Fab antibody fragments which are capable of antigen recognition[1,8-10]. By expressing recombinant immunoglobulin repertoires in phage display libraries, it is possible to enrich and screen millions of prospective clones for antigen recognition[11,12].

Currently there are multiple antibody library constructs which are available through commercial or academic avenues. The two commercially marketed antibody cloning kits are the pCANTAB5E cloning kit (Pharmacia, Piscataway, NJ), which is used to isolate single chain (ScFv) antibodies, and the SurfZAP™ kit (Stratagene, La Jolla, CA), which can be used to clone Fab fragments using methods described elsewhere[13]. We have employed the SurfZAP™ kit with mouse oligonucleotides purchased from Tera Biotechnology (La Jolla, CA) with some success. Human primers can also be purchased directly from Stratagene for use with this kit.

SurfZAP™ uses a lambda viral construct to efficiently infect the antibody genes into the XL-1 Blue cell line. Most academic labs are now shying away from the use of lambda in favor of direct electroporation of the plasmids into the bacterial cells. Two commonly used constructs are the pHen vectors (ScFv) developed by Marks[14] and the pComb3H vectors developed by Barbas[5]. The present focus is the integration of the pComb3H constructs into large scale recombinant antibody production for the development of Fabs which bind *Yersinia pestis* and cholera toxin B with high affinity.

2. MATERIALS AND METHODS

2.1 Toxin Preparations.

Botulinum toxin type A complex (500 kDa) and type B complex (500 kDa) were purchased from WAKO Chemicals (Richmond, VA). The type A and type B neurotoxins (each 150 kDa) were isolated from liquid bacterial culture according to published methods and the neurotoxins were judged pure by SDS-PAGE and amino acid sequence analysis[14]. Toxoid of the type B complex was prepared as reported[15].

2.2   Mouse Library Construction.

2.2.1   Mouse Immunization Protocol for Recombinant Antibodies.

Five female BALB/c mice were immunized subcutaneously with 0.25 ml of pentavalent botulinum toxoid types A, B, C, D, and E (Michigan Department of Public Health), emulsified in one volume Complete Freund's Adjuvant (CFA, Difco, Detroit, Mich.). The mice were subsequently boosted three times at 17 day intervals with 0.25 ml of pentavalent vaccine emulsified in one part Incomplete Freund's Adjuvant (IFA). Five days after the last immunization, the mice were immunized with 50 µg of toxoid of botulinum type B complex in IFA. The mice were then primed with an intravenous injection of 100 ul of botulinum toxin B complex at 2 ng/ml, which also contained 100 µg of botulinum toxoid type B complex. Mice were killed and spleens removed three days after priming immunization. Sera were pooled and analyzed by direct ELISA for titer to botulinum toxin type A and B complexes, with an endpoint titer of 1:100,000.

2.2.2   Anti-botulinum toxin direct ELISA.

Direct ELISA assays were performed using 96 well microtiter plates (Immulon II, Dynatech, Chantilly, Va) coated overnight at 4°C with 100 µl of specified botulinum toxin at 2 µg/ml of phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.4, Sigma, St. Louis, Mo) and with bovine serum albumin (BSA, OEM Concepts, NJ) at the same concentration as the negative antigen. Botulinum type B toxin complex or botulinum type B neurotoxin (NT) were used as antigens in assays to characterize the specificity of recombinant Fabs. The plates were then washed six times using an automated plate washer with PBS Wash Buffer (PBS, 0.1% Tween 20, 0.1% thimerosal, pH 7.4). Sera diluted in ELISA Dilution Buffer (5% Dry Skim Milk, 0.1% Tween 20, 0.001% thimerosal, PBS pH 7.4), undiluted Fab and hybridoma culture supernatants were added to alternating wells of the ELISA plates containing specified botulinum toxin and negative BSA antigen and were incubated for 1 h at 37°C. The plates were then washed as before with ELISA wash buffer. Horseradish peroxidase (HRP) conjugated goat anti-mouse (Kirkegaard and Perry, Gaithersburg, MD) or anti-Fab (Accurate Chemicals, Westbury, NY) antibody diluted 1:2500 in ELISA dilution buffer was added to each well of the ELISA plates and incubated for one hour at 37°C. The plates were washed six times with PBS wash buffer. 2,2'-Azinobis(3-ethylbenzthiazoline-sulfonic acid (ABTS) substrate (Kirkegaard and Perry, Gaithersburg, MD) was added to each well and incubated for one hour at 37°C. The optical density (OD) at 405 nm of each well of the ELISA plate was determined using an ELISA plate reader (Dynatech, Chantilly, Va). An adjusted OD was obtained by subtracting the OD of the reaction of the antibody or Fab with the negative antigen (BSA) from the OD of the reaction of the antibody solution to the positive antigen. The positive cutoff for this assay was calculated from the adjusted OD of the mean plus three standard deviations of three negative controls consisting of culture media without Fab, without monoclonal antibodies, or without botulinum antibody negative mouse sera.

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2.2.3 First Strand cDNA Synthesis and PCR Amplification.

Twenty micrograms of total RNA was isolated as described[16], allowed to anneal with either oligo-dT18 or heavy chain specific immunoglobulin primers, and then extended with 80 units of M-MLV RNase H (Stratagene) and 1mM dNTPs. cDNA was processed and amplified essentially as described[10] in order to isolate individual sets of immunoglobulin genes by PCR amplification.

2.2.4 Construction of primary lambda library.

Heavy and light chain PCR fragments were subjected to digestion with *Sfi I* overnight at 50°C. The digested chains were gel purified and 1 µg of each set were ligated together in a final volume of 10 µl containing 5 units T4 DNA ligase (Gibco/BRL). Two identical ligations were pooled, phenol extracted, ether extracted, and ethanol precipitated. The pellet was resuspended in water and subjected to digestion with 100 units each of *Spe I* and *Not I* (Boehringer-Mannheim) overnight at 37°C. The resulting 1.4 kB insert was gel isolated in 10 µl of water. Forty nanograms of the Fab gene insert was ligated into *Not I/Spe I* digested SurfZAP lambda arms[10,18-21] and packaged into lambda virus[22]. The primary lambda library was titered on XL-1 Blue cells and then amplified using the plate lysate amplification technique[23].

2.2.5 Production of phage for bio-panning.

The resulting primary lambda library was then subjected to mass excision by infection with ExAssist helper phage in the presence of XL-1 Blue cells[22]. The excised phagemid were amplified in SOLR cells in LB broth (100 µg/ml carbenecillin; 50 µg/ml kanamycin) and allowed to amplify for six hours at 37°C. The cells were resuspended in 10 mM $MgSO_4$ and 1 x 10^9 SOLR cells were mixed with 9 x 10^9 pfu of VSCM13 helper phage for 15 min. at 37°C. The culture was then diluted to an $OD_{600}=0.1$ with LB$^{carb/kan}$ broth and shaken at 30°C overnight. The culture was centrifuged and the supernatant containing the bacteriophage was precipitated with polyethylene glycol 8000 (PEG)[4]. The pellet was resuspended in 1 ml of Tris-EDTA (TE) buffer and PEG precipitated. The bacteriophage pellet was resuspended in 100 µl of TE/0.1% BSA.

2.2.6 Bio-panning enrichment and rescue of positive clones.

The bio-panning procedure used was a modification of two protocols[9,10]. The wells of Nunc 96 well Immuno-plates were coated for 2 h at 4°C with 100 µl of 10 µg/ml botulinum type B complex or NT in 100 mM sodium bicarbonate buffer (pH 9.0). The plates were then blocked with 1% BSA, 0.5% Tween-20, in PBS (blocking buffer) for 30 min. at 25°C, followed by three washes with 0.5% Tween-20/PBS (wash buffer). Approximately 2 x 10^{10} cfu phagemid added to

each well were incubated for 2 h at 4°C. Unbound phagemids were removed by washing once with wash buffer. To avoid the loss of rare clones, the first round of bio-panning was washed only once and subsequent rounds were washed two and ten times. The bound phagemids were eluted by adding 100 µl of 100 mM glycine/0.5% BSA (pH 2.5) to the well and incubated for 10 min. at 25°C. The elution mix was then mixed briefly and neutralized with 2 M Tris base. Eluted phage were used to infect XL-1 Blue cells and the sample was diluted with LB ^{carb} and shaken for 1 h at 37°C. VCSM13 helper phage (1 x 10^{10} pfu) and 50 µg/ml kanamycin were added and the culture shaken overnight at 29°C. The phagemids were collected as above and the bio-panning was repeated for a total of three rounds of selection.

2.2.7 Colony lift hybridization.

Following the final round of enrichment, the positive colonies were duplicate plated on LB ^{carb} plates using wetted nitrocellulose filters overlaid onto the bacterial plates. One set of colonies growing directly on the nitrocellulose filters was lysed in a sealed chamber containing a 0.25 inch layer of chloroform on the bottom. The filters were submerged in lysozyme buffer (50 mM Tris {pH 8.0}, 150 mM NaCl, 5 mM MgCl$_2$, 3% BSA, 400 µg/ml lysozyme, and 1 U/ml DNase I). After 1 h at 25°C, the filters were transferred to a fresh bath of lysozyme for an additional hour, then washed twice for 10 minutes each, in TBST (20 mM Tris {pH 7.5}, 150 mM NaCl, 0.05% Tween-20) and blocked for one hour in blocking solution (1% BSA, 20 mM Tris, 150 mM NaCl). Finally, the filters were transferred to 125 ml of blocking solution to which 160 µl of 200 nM ^{125}I labeled-botulinum type B toxin complex (ICN Industries, Irvine, CA) or ^{125}I labeled-NT had been added. Toxins were labeled using the Chloramine T method and unbound ^{125}I was removed by gel filtration on a Sephadex G-10 column run in 0.05 M acetic acid, 0.2 M NaCl, 0.3% BSA. After 1.5 h at 25°C, the unbound labeled antigen was removed by six 10 minute washes with TBST. The filters were then air dried on blotting paper and exposed to autoradiograph film for 4 h. Re-alignment of labeled colonies with the master plate allowed the identification of bacterial clones which express antibodies capable of binding antigen.

2.3   Fab Clone Analysis.

2.3.1   ELISA of bacterial supernatants.

Overnight cultures of positive clones were grown and induced with 1 mM isopropyl -D-thiogalactopyranoside (IPTG) when in early log phase. The cells were pelleted and 100 µl of the supernatant was allowed to bind for 1 h to microtiter plates that had been coated with 100 µl of 5 µg/ml botulinum type B complex or NT. The plates were blocked and washed as described above. The wells were then probed with 100 µl of goat anti-mouse Fab alkaline phosphatase antibody, which served as the secondary antibody. The conjugate, allowed to bind for 2 h, was developed after six wash cycles.

2.3.2 Subcloning into pHist.

The phagemid vector pSurfscript was cleaved with *Not I/Spe I* and the Fab gene insert was excised in preparative scale in a 2% TAE gel[10,18]. The insert was then ligated into *Not I/Spe I* cut pHist vector. pHist is a modified version of pSurfscript which has a linker encoding six histidines inserted in place of the cpIII gene.

2.3.3 Purification of Fab.

Whole cell extracts were prepared by sonication in 2-(N-Mopholino)ethanesulfoninc acid (MES) buffer {pH 6}. Filtered extracts were passed over a 5 ml Hi-trap™ SP cation exchange column and eluted in a linear NaCl gradient in MES. The pooled fractions were subjected to metal chelate affinity chromatography under non denaturing conditions according to the manufacturer (Qiagen) and Lindner[25].

2.3.4 BIAcore Kinetic Analysis.

Fab was dialyzed into 5 mM sodium maleate buffer (pH 5.8). All solutions were filtered through a 0.22 μm filter and/or degassed. Fab was covalently immobilized by amine coupling (cat# BR-1000-50) using N-hydroxysuccinimide esters to immobilize 2000 RUs of antibody to a CM5 sensor chip. Bot toxoid type B (M.W. 500Kd) in HBS (10 mM HEPES {pH 7.4}, 150 mM NaCl, 3.4 mM EDTA, 0.0005% Surfactant P 20) was passed over the chip at 30 μl/min using indicated concentrations. Kinetic determination was performed using BIA Evaluation 2.1 and values were averaged constants from two separate immobilizations.

3. RESULTS

Regardless of which constructs were used to produce a combinatorial antibody library, the bio-panning selection was basically the same. Filamentous bacteriophage were produced by co-infection of the XL-1 Blue with the VSCM13 helper bacteriophage. The VSCM13 helper phage carried a copy of the gene III protein which had been deleted from the antibody construct such as pComb3H. The co-infection resulted in the production of bacteriophage which displayed the Fab protein as a fusion with the cpIII coat protein. Coat protein III was present at 3-5 copies per virus and played a role in the infection of *E. coli*. The Fab was fused to less than one cpIII protein per virus and therefore the phage were still infective while presenting the recombinant gene.

In order to select for phage containing an antigen reactive clone it was necessary to immobilize the antigen of interest on a solid support such as an ELISA well. We found Nunc Maxi and ImmunoSorp plates to be good binders of a wide variety of protein preparations. Costar #3690 are recommended by some labs although these plates have smaller capacities (200 ul) and are difficult to adapt to automatic ELISA plate washers. It is sound practice to confirm the

immobilization of the antigenic extract to the microtiter plate by ELISA which may utilize a polyclonal or monoclonal serum.

Following bio-panning enrichment of a botulinum responsive library, screening with radiolabeled botulinum type B complex revealed numerous positive clones. Forty eight were confirmed by ELISA and were subcloned into the pHist purification vector. In the pHist vector the Fab heavy chain gene was expressed as a fusion with a histidine hexapeptide tail, rather than as a fusion with the cpIII protein. Commercially available Ni-NTA resins bind histidine oligomers with high affinity allowing for affinity purification of the Fab protein.

Antibody protein from a whole cell extract was first bound to a Hi-trap SP cation exchange resin and eluted with a 0 to 500 mM NaCl gradient. The pooled fractions were then adjusted to pH 8 and allowed to bind Ni-NTA resin in batch. The antibody was then easily eluted under non reducing conditions with 500 mM imidazole.
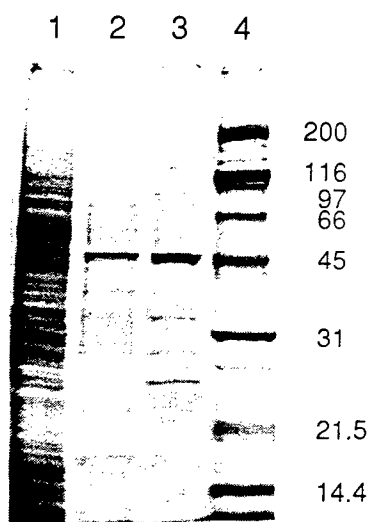
Figure 1: 12% SDS-PAGE analysis of purified Fab proteins stained with coomassie blue. Lane 1: Bio-Rad Low molecular weight markers, Lane 2: Crude cell lysate, Lane 3: Pooled cation column fractions, Lane 4: Pure anti-BotFab, Lane 5: Broad M. Wt. markers.

Figure 2: Western Blot Analysis of Purified Fab Proteins. Five microliters of purified Fab protein was separated on a 12% SDS gel under non-reducing conditions (lane 1) or in the presence of a reducing agent (lanes 2 and 3). The proteins transferred to nitrocellulose were probed with either rabbit anti-mouse Fab (lane 1) or with rabbit anti-mouse kappa light chain antibodies. All lanes were then probed with goat anti-rabbit conjugated with alkaline phosphatase as a secondary antibody. Lane 1: 5 μl pHist 5 purified Fab protein. Lane 2: 5 μl pHist 5 purified Fab protein. Lane 3: 100 ng of a purified unrelated monoclonal antibody known to have a kappa light chain.
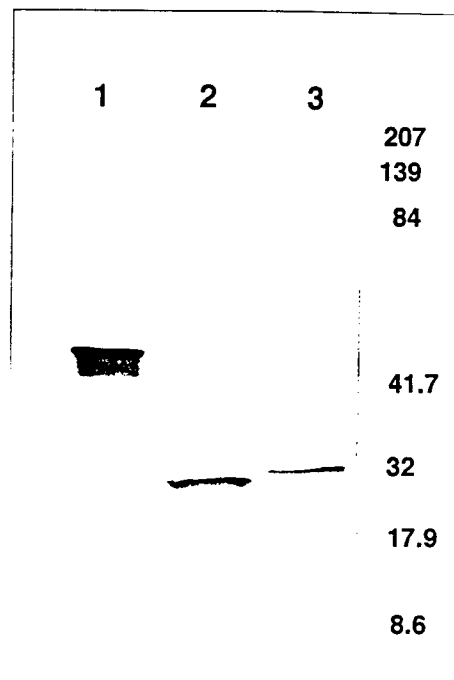
Western blot of SDS-PAGE gels run under non reducing conditions and probed with mouse anti-Fab antiserum confirmed that the kDa polypeptide was mouse Fab (Figure 2, lane 1). Western blot analysis of the purified Fab protein under reducing conditions using mouse anti-kappa light chain antiserum confirmed that the light chain was the 31 kDa polypeptide (Figure 2, lane 2). The results demonstrated that, using shaker flasks, bacterially expressed mouse Fab could be isolated in a highly purified form at 1.6 mg/liter using LB broth.

DNA was prepared from single clones and subjected to dideoxy nucleotide sequencing in order to compare the expressed proteins. Four clones were compared and found to vary in their DNA sequence, but not in their amino acid sequence. Variations in codon usage in the wobble position of each triplet codon showed that each clone was unique but that the amino acid sequence remained the same. This could indicate a strong selective pressure to preserve the heavy and light chain combination. The sequencing also confirmed that six histidines were successfully added to the heavy chain of the antibody for use in affinity purification. The addition of the oligomer to the carboxyl terminus did not adversely affect binding of the Fab to the target antigen.

Competitive ELISA indicated that binding of the Fab was highly specific and had a potential equilibrium binding constant in the nanomolar range. To more accurately determine the kinetics of binding, BIAcore analysis (Pharmacia Biosensor, Piscataway, N.J.) was performed. The BIAcore allows for the real time assessment of binding characteristics as measured by surface plasmon resonance. BotFab 5 was immobilized to a sensor chip and botulinum toxoid type B was passed over the antibody. Figure 3 shows a sensoragram which reflects the binding and dissociation reaction versus time.

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Kinetic Analysis of Anti-Bot Fab
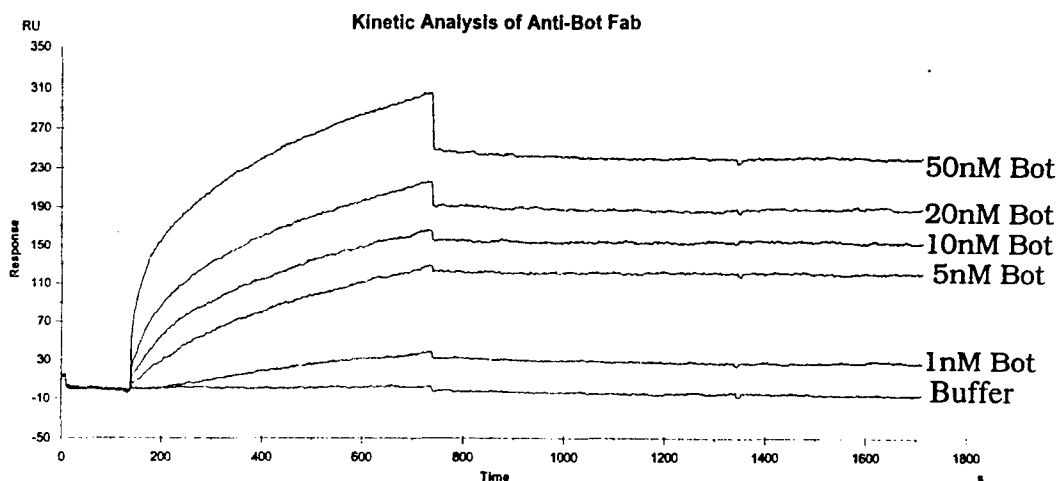
Figure 3: BIAcore Kinetic Analysis of anti-Bot Fab. Fab at 50 µg/ml was dialyzed into 5 mM maleate buffer (pH 5.8) and immobilized to a CM5 sensor chip using amine coupling. Bot toxoid B at 50nM, 20nM, 10nM, 5nM, and 1nM were passed over the surface at 20 µl/min for 750 seconds. The binding reaction is indicated by the response (RU) and was used to calculate the k_a. A dissociation phase of 2 hours was performed by washing with buffer containing no toxoid. The abrupt drop at 750 seconds was due to a change in the bulk refractive index as the toxoid was replaced with HBS buffer. The k_d was calculated from this dissociation period using BIAevaluation 2.1 (see materials and methods).

To determine the on and off rates of the antibody a comparison of the resonance unit (RU) signal over time using various toxoid concentrations was performed. The data in figure three indicates an on rate of 5×10^5 M/sec and an off rate of 1.6×10^{-5} M^{-1}. The ratio of k_d and k_a gives and equilibrium constant of 32 picomolar indicating a tight binding affinity. The stability of this interaction has prompted ERDEC to file for a patent of this cell line for potential use as a detection reagent and to deposit the cell line in the ATCC repositories (Accession number ATCC 98316).

4. DISCUSSION

The need for a more economical means of mass producing antibodies for detection of biological warfare agents was addressed by the selection of recombinant antibody fragments (Fabs) from combinatorial libraries. The development of this technology is advantageous for rapid, large scale production because it permits the use of standard biomanufacturing techniques using bacterial fermentation.

The ability to alter the selection process gives an additional level of control which allows the selection process to be driven towards a particular antigen or epitope[12]. Antibody producing

clones are available in prokaryotic systems as a double stranded phagemid; the genetic manipulation of this plasmid DNA is straightforward and provides the capability to alter the affinities by mutagenesis or by chain shuffling[14,26].

Downstream processing can be simplified by inserting affinity tags at the nucleotide level, allowing the Fab to be expressed as a fusion with a histidine hexapeptide tag on the carboxyl terminus. Detection sensitivity and speed can be further enhanced by adding reporter proteins compatible with detection systems directly into the expression construct, thus eliminating the need for an additional conjugation step. These constructs can be produced in various expression systems such as CHO cells, baculoviral systems, or secreted in the milk of transgenic animals, which may increase yields.

Requirements for antibodies with particular specificities and affinities have not been met using standard technologies[27]. Biosensor applications and catalytic immuno-enzyme assays require antibodies with specific epitope recognition and affinities, which can be best produced by a recombinant antibody approach. Bacterial expression of Fab fragments gives lower yields than hybridoma cultures, but is far less expensive to scale up. Large scale growth and purification of cell lines is faster, giving a finished product in as little as one week. The power of this technique lies in the vast numbers of clones that can be screened and enriched in order to isolate unique specificities and in the ease with which these clones can be genetically manipulated. As this technique gains wider acceptance for use in isolating immunoglobulins, significant improvements will give the researcher increased flexibility in immunodiagnostics and therapeutics.

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