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Selective improvement of tumor necrosis factor capture in a cytokine hemoadsorption device using immobilized anti-tumor necrosis factor

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Abstract

Sepsis is a harmful hyper-inflammatory state characterized by overproduction of cytokines. Removal of these cytokines using an extracorporeal device is a potential therapy for sepsis. We are developing a cytokine adsorption device (CAD) filled with porous polymer beads which efficiently depletes middle-molecular weight cytokines from a circulating solution. However, removal of one of our targeted cytokines, tumor necrosis factor (TNF), has been significantly lower than other smaller cytokines. We addressed this issue by incorporating anti-TNF antibodies on the outer surface of the beads. We demonstrated that covalent immobilization of anti-TNF increases overall TNF capture from 55% (using unmodified beads) to 69%. Passive adsorption increases TNF capture to over 99%. Beads containing adsorbed anti-TNF showed no significant loss in their ability to remove smaller cytokines, as tested using interleukin-6 (IL-6) and interleukin-10 (IL-10). We also detail a novel method for quantifying surface-bound ligand on a solid substrate. This assay enabled us to rapidly test several methods of antibody immobilization and their appropriate controls using dramatically fewer resources. These new adsorbed anti-TNF beads provide an additional level of control over a device which previously was restricted to nonspecific cytokine adsorption. This combined approach will continue to be optimized as more information becomes available about which cytokines play the most important role in sepsis.

Keywords

sepsis; hemoadsorption; cytokine; antibody; TNF

INTRODUCTION

Severe sepsis, defined as acute onset organ failure in the setting of infection, is the leading cause of death in noncoronary intensive care units in the US and costs billions of dollars per year.¹ Sepsis is characterized by the overproduction of several different types of

inflammatory mediators, which has been identified as one possible cause of the systemic inflammation and organ dysfunction seen in septic patients.^{2,3} An increase in the understanding of the immune response in sepsis^{4,5} has led to expanded research into blood purification therapies intended to reduce the concentration of circulating inflammatory mediators.^{6,10} The usefulness of blood purification, either filtration or adsorption of blood or plasma proteins, as a treatment for sepsis lies in its ability to remove a broad range of molecules rather than targeting single mediators.

Our group is currently developing a hemoadsorption device to treat severe sepsis by depleting circulating levels of pro- and anti-inflammatory cytokines. This cytokine adsorption device (CAD) consists of a column packed with biocompatible CytoSorbTM polymer beads (CytoSorbents, NJ), whose effectiveness for cytokine removal has been demonstrated in both *in vitro* and *ex vivo* experiments.^{11,12} We have modeled the performance of the beads and the device based on data obtained during scaled-down cytokine capture experiments for the cytokines interleukin-6 (IL-6), tumor necrosis factor (TNF), and interleukin-10 (IL-10). Our results indicate that the beads remove almost 90% of middle-molecular weight proteins such as IL-6 and IL-10 (18–21 kDa) after 4 h, but only 50–60% of the relatively large TNF trimer (52 kDa).¹³ This result is not surprising due to the limited range of pore sizes available on the surface of the CytoSorbTM beads: the pores are designed to exclude larger molecules such as albumin (66 kDa) and fibrinogen (340 kDa). Lixelle (Kaneka Corporation, Osaka, Japan), an alternative adsorbent material being tested to treat hypercytokinemia, has shown only 20% removal of TNF in 4 h using the same experimental setup as with the CytoSorbTM beads (unpublished data). Our conclusion is that beads which target cytokines nonspecifically are not capable of removing TNF at comparable levels to smaller cytokines while maintaining their ability to exclude larger proteins.

Increasing removal of TNF within our device is of particular interest, as sustained high concentrations of TNF are negatively correlated with survival in septic patients.¹⁴ Neutralization of TNF in small animal sepsis models using soluble receptors and monoclonal antibodies has been shown to reduce mortality^{15,16} and several candidates from each category of TNF-specific antagonists have been tested in clinical trials since 1993. A review of these trials demonstrates that no statistically significant improvement in patient mortality has been observed; in some cases, survival rates were actually significantly better in the placebo group.¹⁷ Many argue that these therapies have failed because they make no distinction between patients requiring immune suppression and those requiring immune augmentation, due to issues such as type of infection, timing, and severity of insult.^{18,19} Our approach currently provides for either type of immunomodulation for smaller proinflammatory and anti-inflammatory cytokines. We hypothesized that a combined approach of specific and non-specific cytokine capture would selectively increase capture of TNF to levels comparable to those of other cytokines, thus further increasing the efficacy of our device.

The main goal of this study was to accelerate the rate of removal and overall capacity for TNF capture by immobilizing anti-TNF on the outer surface of the beads in the CAD. We explored covalent versus passive immobilization techniques as well as several surface functional group amplification methods, including poly-L-lysine (PLL) cross-linking. We have also developed a simple method of bound antibody quantification which dramatically decreased the amount of time and resources involved in quantifying antibody binding for the various immobilization schemes. Passive adsorption of anti-TNF led to a 29% increase in TNF removal over covalent binding and a 43% increase over unmodified CytoSorbTM beads. Lastly, we characterized the retention of the passively adsorbed antibodies to suggest the clinical safety of treatment with a CAD containing adsorbed anti-TNF beads.

MATERIALS AND METHODS

Either goat anti-human IgG-horseradish peroxidase (HRP) conjugated antibodies, rat anti-human TNF antibodies, or rat IgG antibodies were used depending on the type of experiment that was done (Invitrogen, Carlsbad, CA). All chemicals used during antibody immobilization steps were from ThermoFisher (Pittsburgh, PA) unless otherwise stated.

Bead modification and EDC activation

The CytoSorb™ beads are made up of a polystyrene-divinylbenzene (PSDVB) copolymer with a biocompatible polyvinylpyrrolidone (PVP) coating. The beads range in size from 300–800 μm with an average diameter of 533.2 μm and a pore size range of approximately 8–50 $\text{\AA}$.

The chemistry chosen for initial modifications to the Cyto-Sorb™ beads was based on that of Zammattéo et al. for modifying polystyrene microwells.²⁰ The CytoSorb™ beads were modified using a ring-opening chemistry which incorporates carboxyl groups into the backbone of the polymer (Figure 1). Note that in Figure 1 the PVP coating is not present as it does not participate in the carboxylation. Potassium permanganate, KMnO_4 , (5 g dissolved in 100 mL 1.2N H_2SO_4) was used as the oxidizing agent. The reaction took place for 2 h at 60°C and excess manganese oxide was rinsed off using 6N hydrochloric acid (HCl). At that point, beads were assayed for surface density of carboxyl groups. The modified beads were incubated overnight with a solution of 0.01M dicyclohexylcarbodiimide (DCC) and 0.01 M para-nitrophenol (PNP) in pyridine, which crosslinked the bright yellow PNP molecule with the carboxylated polymer. Unreacted PNP was washed off with tetrahydrofuran and the beads were incubated for 15 min with NaOH to release nitrophenoxide into solution. The amount of carboxyl groups (corresponding to the total molar amount of nitrophenoxide in solution per unit mass of polymer) was then determined via UV/Vis spectroscopy and Beer's Law using the known extinction coefficient for nitrophenoxide, $1.57\text{E}4 \text{ M}^{-1} \text{ cm}^{-1}$.²¹ Three measurements each of three independent bead modifications were taken and averaged to get the average carboxyl group density of the beads.

Those modified beads not used in the carboxylation assay were activated using the well-characterized 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) chemistry (Figure 2).²² The beads were incubated for 1 h at room temperature with a solution of 26 mg EDC (Sigma, St. Louis, MO) per gram of beads dissolved in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 4.5. Activation was terminated by rinsing several times with MES buffer and DI water. To complete the immobilization, the activated beads were incubated with a 40 μg antibodies per gram of beads of the in 0.1 M sodium phosphate buffer at pH = 7.0. The process was completed when remaining active sites were blocked using a solution of phosphate buffered saline (PBS) and 0.1% v/v Tween 20. Following immobilization of the antibodies, beads were washed several times with 1.0 M NaCl. The antibody-immobilized beads were then preserved at 4–8°C in a solution of deionized water and 0.01% v/v thimerosal for up to 24 h before being packed into devices for cytokine capture experiments or assayed for anti-IgG-HRP concentration.

Passive adsorption of antibodies was carried out by simply incubating unmodified beads with 40 μg antibodies per gram beads for a total of 2 h at room temperature in sodium phosphate buffer. The washing steps following antibody incubation were performed as described above. Beads containing anti-TNF or IgG were then used in cytokine capture experiments while those containing anti-IgG-HRP were assayed for HRP concentration.

Poly-L-lysine immobilization and antibody crosslinking

PLL is a high molecular-weight linker molecule containing many exposed amine groups, ideal for increasing the functionality of the bead surface. PLL can be covalently bound to the carboxylated beads via a peptide bond and subsequently cross-linked using glutaraldehyde to terminal amines on the antibodies. This crosslinking was accomplished using a modified version of the protocol found in Zhang et al.²³ Prior to immobilization, beads were carboxylated and activated using EDC according to the previously described procedure. Once activation was complete, beads were washed very quickly with MES buffer and water. The bead samples were then incubated with 0.4 mg PLL (150–300 kDa, Sigma) per gram of beads for 4 h at room temperature. Beads were washed once again with water and sodium phosphate buffer. Next the beads were incubated for 1 h with a 10% glutaraldehyde solution and washed thoroughly. As mentioned above, 40 µg antibodies per gram of beads were then added during the immobilization step and incubated for 2 h, followed by a brief incubation with a solution of 0.2 M glycine and 0.01 M cyanoborohydride. Beads were then washed one final time before being assayed for anti-IgG-HRP concentration or packed into a device for cytokine capture. In each case, three independent immobilizations were carried out and HRP activity was measured three times for each immobilization.

Anti-IgG-HRP assay

The high cost of anti-TNF and time involved in cytokine capture and ELISA make characterizing the efficiency of immobilization techniques cumbersome. The following enzyme-based assay is a more direct quantification technique and dramatically reduces the amount of time and resources involved. Although the theory behind this assay is based on that of solid-phase enzyme-linked immunosorbent assay (ELISA), this particular protocol is especially similar to that of Mansur et al.²⁴ Beads containing immobilized or adsorbed anti-IgG-HRP antibodies were split into three or more samples of approximately 0.2–0.5 g each. Beads were transferred using a custom-made filtered suction tool which allowed for dehydration of the beads while simultaneously ensuring no beads were lost or broken during transfer. Bead samples were placed in glass test tubes and resuspended with a standard ELISA buffer solution. Anti-IgG-HRP standards from 1000 pg/mL to 0 pg/mL were pipetted in duplicate into an empty polystyrene 96-well plate (Nalgene, Rochester, NY). Tetramethylbenzidine (TMB) substrate solution was added in equal proportions to both the microwells containing standards and the test tubes containing the bead samples. The HRP was allowed to react with the TMB for 20 min in the dark. Sulfuric acid was then added to the wells and test tubes, again in equal proportions, to stop the enzyme-substrate reaction. A small amount of liquid from the test tubes containing bead samples was then diluted with deionized water prior to being pipetted in duplicate into microwells. The optical density was then read using a ThermoFisher MultiSkan optical density reader at 450 nm. HRP concentration in the bead samples was determined using the anti-IgG-HRP standard curve. Values for moles of antibody per mass of beads were determined after measuring the mass of the dry beads, again using the suction method for transferring beads. A paired student's *t*-test was used to test for significant differences in surface antibody concentration per mass of beads between samples, with $p < 0.05$ denoting a statistically relevant difference. Three of these assays were carried out for each type of bead tested.

Adsorbed antibody retention

Anti-IgG-HRP antibodies were passively adsorbed onto Cyto-SorbTM beads using the aforementioned protocol. Beads were then packed into a 1 mL CAD and perfused continuously for 2 h at a flow rate of 3.2 mL/min with a 40 mL solution of PBS containing 50 mg/mL bovine serum albumin (BSA). Samples of the solution were taken over time and assayed using the anti-IgG-HRP direct quantification method previously described. Three trials of this experiment were performed. This same procedure was also carried out as a

preparatory step for one set of experiments using adsorbed anti-TNF beads prior to performing cytokine capture as described below.

Cytokine capture during recirculation

The cytokine adsorption device (CAD) consisted of a cartridge filled with CytoSorb™ polymer beads. Beads were contained within the CAD by two polypropylene mesh screens with a nominal pore size of 149 µm. The other components for the CADs were obtained from Supelco (Sigma) and each device contained ~1.5 g of polymer.

For each recirculation experiment, CADs were packed with polymer and connected in line with a peristaltic pump (Figure 3). The inlet and outlet tubing ports were connected to a reservoir containing 8 mL of horse serum or PBS spiked with one or more cytokines. The reservoir fluid was pumped through the CAD at a flow rate of 0.8 mL/min and samples were taken from the reservoir at times $t = 0, 15, 30, 60, 90, 120, 180,$ and 240 min. Samples were stored at -70°C for subsequent assay using ELISA. Concentrations of IL-6, IL-10, and TNF were determined using BioSource ELISA kits (Invitrogen) according to the instructions of the manufacturer. Once again, a paired student's t -test was used to test for significant differences in overall cytokine capture after 4 h, with $p < 0.05$ denoting a statistically relevant difference. Three trials of cytokine capture were performed with each type of beads for every experiment.

RESULTS

CytoSorb™ beads were chemically modified to introduce carboxyl (COOH) functionality using potassium permanganate-mediated oxidation. The average surface density of COOH groups achievable on CytoSorb™ beads was 5.17 µmol COOH/g polymer, nearly 100 times less than the degree of carboxylation reported for pure polystyrene by Matteucci and Caruthers.²¹

To expedite testing of the modified beads, we used a direct assay technique to quantify antibody concentration on a solid matrix. An enzyme-based antibody assay was used to compare levels of bound or adsorbed antibodies for three different immobilization methods: passive adsorption, EDC-mediated immobilization on carboxylated beads, and PLL-crosslinked beads. The passive adsorption beads showed significantly higher levels of adsorbed antibody compared with both the EDC-mediated and PLL-crosslinked covalently bound antibodies: $9.6 \pm 0.46 \times 10^{-12}$ mol Ab/g beads versus $8.0 \pm 0.41 \times 10^{-13}$ mol Ab/g beads and $2.9 \pm 0.031 \times 10^{-12}$ mol Ab/g beads, respectively (Figure 4).

The results of the enzyme-based assay were confirmed using cytokine capture. Rat anti-human TNF antibodies were immobilized on the modified beads via a carboxyl-amine bond using EDC activation chemistry as well as passive adsorption onto unmodified beads. The ability of both the covalent and adsorbed anti-TNF beads to capture TNF from horse serum during 4 h of recirculation was then quantified and compared with that of the unmodified beads. These results can be seen in Figure 5; the adsorbed antibody beads removed 99% in 4 h while the covalently bound anti-TNF beads and unmodified beads removed only 69 and 55%, respectively. PLL cross-linked beads showed the same level of TNF capture as the covalently bound anti-TNF beads (data not shown).

We also performed TNF capture using beads containing adsorbed IgG antibodies which were not specific to TNF to demonstrate the specificity of the adsorbed antibodies. Figure 6 shows these results compared with TNF capture using adsorbed anti-TNF and unmodified beads. The beads containing IgG antibodies removed only 37% of TNF, while the unmodified beads removed 55% and the adsorbed anti-TNF beads removed 98%. Lastly, we

determined whether the adsorbed antibodies had any effect on capture of other, smaller cytokines. We performed multicomponent capture of IL-6, IL-10, and TNF from serum using unmodified beads and adsorbed anti-TNF beads. These results can be seen in Figure 7; overall TNF capture after 4 h improved 33% with no statistically significant difference in IL-6 or IL-10 capture in the presence of the anti-TNF antibodies.

We measured leaching of passively adsorbed antibodies from the beads during consecutive 1-h recirculations, each with a fresh PBS/BSA solution, to characterize the stability of passively adsorbed anti-TNF. Figure 8 shows the results; 59.4 and 8.8% of the initial amount of adsorbed anti-IgG-HRP leached off in the first and second hour of buffer recirculation, respectively. The rate of antibody leaching dropped from a maximum of 7750 pg antibody leached/min to 215 pg/min. This same 2 h flushing procedure was carried out for beads containing adsorbed anti-TNF. We performed several trials of TNF capture using anti-TNF beads that had been prepared with and without the preflush step and saw no difference in TNF capture ability between the two types of beads (Figure 9).

DISCUSSION

Our goal in this study was to demonstrate that TNF capture in our cytokine adsorption device (CAD) can be improved by incorporating anti-TNF antibodies on the adsorbent polymer in the device. We tested several types of immobilization methods and found that, due to the highly hydrophobic nature of the beads, passive adsorption results in a 12-fold increase in surface antibody coverage than covalent immobilization. Using the passively adsorbed anti-TNF beads, overall TNF capture after 4 h was increased by over 30%. Multicomponent capture experiments demonstrated that the presence of the anti-TNF results in less than a 10% decrease in total amount of IL-6 or IL-10 removed from solution over four hours of recirculation. The abundance of surface area on the inner surfaces of the beads ensure that other proteins can still adsorb effectively without interfering with the specific binding of TNF occurring at the outer layer of the beads.

Our initial goal was to covalently bind the antibodies to the beads. We used a carboxylation method intended for pure polystyrene on the polystyrene-divinylbenzene Cyto-Sorb™ beads, which resulted in a two order of magnitude decrease in carboxylation of our beads versus pure polystyrene.²¹ This low level of modification was most likely due to the PVP coating on the beads. EDC-mediated activation of the carboxylated beads showed only a 15% increase in overall TNF capture after 4 h. We attempted to use PLL to amplify the number of functional groups on the surface but saw no significant change in TNF capture compared to the direct covalent linkage. One gram of beads has a total surface area of 850 m², only 0.088 m² of which is exposed outer surface area. The carboxylation procedure does not distinguish between the inner and outer bead surfaces, meaning that only about 1/10,000 of the total amount of functional groups is available for antibody or PLL binding. This factor, combined with the low level of carboxylation, results in the lack of effectiveness of covalent binding, even with the PLL amplification. We hypothesize that the modification of the beads for covalent binding alters the hydrophobicity of the beads, resulting in lower levels of nonspecifically adsorbed antibodies as well.

We have also shown that the rate of leaching of the passively adsorbed antibody can be reduced to 300 pg/min after a 2 h flush in a 1.5 g CAD without any loss to functionality of the beads. Our conclusion is that the antibodies leaching off during the flushing were actually loosely adsorbed upon surface-bound antibodies that do not leach off. Therefore, the monolayer of antibodies that remains is still able to retain the cytokine capture ability of the stacked antibody configuration. For a 4 h clinical treatment with a 500 g cartridge, the total amount of antibody leached following the 2-h flush would correspond to ~24 µg. This

value is well within a safe range for clinical purposes when considering that similar monoclonal anti-TNF antibodies which are FDA approved for the treatment of autoimmune diseases such as rheumatoid arthritis and Crohn's disease are given in doses ranging from 3 to 10 mg/kg.²⁵⁻²⁷

We wanted to demonstrate that the effect due to anti-TNF coated beads was the result of a specific interaction between TNF and anti-TNF to justify the use of monoclonal anti-TNF (\$75/100 µg) over a more inexpensive protein. To do so, we immobilized polyclonal IgG antibodies that were not specific to TNF on the beads and performed TNF capture. TNF capture experiments done with the beads containing adsorbed IgG showed a 60 and 18% decrease in TNF removal compared with the adsorbed anti-TNF and unmodified beads, respectively. We attribute this effect to diffusion hindrance caused by the antibodies which prevents adsorption of TNF to the bare polymer surfaces.

We developed a novel assay method for quantifying protein attachment to a solid matrix using an enzyme reaction and subsequent color change to directly detect the immobilized antibody concentration. This method is similar to that described in Mansur et al.²⁴; however, Mansur et al. added the enzyme-conjugated antibodies to microwells containing premassed samples of modified silica gel without washing the samples before adding the substrate which may result in a significant nonspecific signal. Our method avoids this issue by eliminating the need for extra washing steps as all samples are thoroughly washed prior to their being assayed. The main advantages of this assay are the antibodies, which are much less costly than anti-TNF, and the time involved in performing the assay, which is under 30 min. Although the assay itself does not distinguish total antibody concentration from functional antibody concentration (i.e., those oriented with their antigenic site exposed), this assay is a much more direct way of qualifying antibody coverage than basing such measurements on cytokine capture results. The activity of the enzyme may be affected by adsorption to the beads, but any decrease in enzyme activity due to adsorption would have been most evident in the adsorption-only beads. In fact these beads showed significantly higher levels of anti-IgG-HRP, indicating that enzyme activity was not significantly affected by adsorption to the beads. We also assume that any effects from antibody or enzyme aggregation during the adsorption or immobilization steps would occur in all bead types equally and thus not affect the overall results. In the future, we will optimize the anti-TNF adsorption method using our newly developed anti-IgG-HRP assay.

The CAD originally used only nonspecific adsorption to deplete a wide range of middle molecular weight cytokines but was unable to effectively remove TNF. The inclusion of specific antibody-mediated cytokine capture provides an additional level of control over cytokine removal in our device and depletes over 99% of TNF in 4 h. These improvements can be incorporated into the existing extracorporeal setup without adjusting the current proposed treatment scheme of 4 h of continuous hemoperfusion. In addition to using this new antibody adsorption technique to dramatically improve TNF capture, we can also investigate ligands specific for other molecules which may play pivotal roles in sepsis. Our colleagues are developing a mathematical model of sepsis which simulates the time course of sepsis in a patient and tracks probability of survival as a function of various inflammatory mediators.²⁸ This model will be used to determine which factors are most strongly associated with damage in patients with sepsis, and we can use this information to customize the selective cytokine removal feature of our device for more effective removal of those factors.

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References

1. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. *Crit Care Med*. 2001; 29:1303–1310. [PubMed: 11445675]
2. Nylen ES, Alarifi AA. Humoral markers of severity and prognosis of critical illness. *Best Pract Res Clin Endocrinol Metab*. 2001; 15:553–573. [PubMed: 11800523]
3. van der Poll T, van Deventer SJ. Cytokines and anticytokines in the pathogenesis of sepsis. *Infect Dis Clin North Am*. 1999; 13:413–426. [PubMed: 10340175]
4. Cross AS, Opal SM. A new paradigm for the treatment of sepsis: Is it time to consider combination therapy? *Ann Intern Med*. 2003; 138:502–505. [PubMed: 12639085]
5. Baue AE. Multiple organ failure, multiple organ dysfunction syndrome, and systemic inflammatory response syndrome. Why no magic bullets? *Arch Surg*. 1997; 132:703–707. [PubMed: 9230852]
6. Bellomo R. Continuous hemofiltration as blood purification in sepsis. *New Horizons*. 1995; 3:732–737. [PubMed: 8574604]
7. Heering P, Morgera S, Schmitz FJ, Schmitz G, Willers R, Schultheiss HP, Strauer BE, Grabensee B. Cytokine removal and cardiovascular hemodynamics in septic patients with continuous venovenous hemofiltration. *Intensive Care Med*. 1997; 23:288–296. [PubMed: 9083231]
8. Kellum J. Immunomodulation in sepsis: The role of hemofiltration. *Minerva Anesthesiol*. 1999; 65:410–418. [PubMed: 10394812]
9. Kellum JA, Johnson JP, Kramer D, Palevsky P, Brady JJ, Pinsky MR. Diffusive vs. convective therapy: Effects on mediators of inflammation in patient with severe systemic inflammatory response syndrome. *Crit Care Med*. 1998; 26:1995–2000. [PubMed: 9875910]
10. Oda S, Hirasawa H, Shiga H, Nakanishi K, Matsuda K-I, Nakamura M, Ikeda H, Sakai M. Cytokine adsorptive properties of various adsorbents in immunoadsorption columns and a newly developed adsorbent: An in vitro study. *Blood Purif*. 2004; 22:530–536. [PubMed: 15583478]
11. Song M, Winchester J, Albright RL, Capponi VJ, Choquette MD, Kellum JA. Cytokine removal with a novel adsorbent polymer. *Blood Purif*. 2004; 22:428–434. [PubMed: 15316198]
12. Peng Z, Carter M, Kellum J. Effects of hemoabsorption on cytokine removal and short-term survival in septic rats. *Crit Care Med*. 2008; 36:1573–1577. [PubMed: 18434884]
13. DiLeo MV, Fisher JD, Federspiel WJ. Experimental validation of a theoretical model of cytokine capture using a hemoabsorption device. *Ann Biomed Eng*. 2009; 37:2310–2316. [PubMed: 19680812]
14. Casey LC, Balk RA, Bone RC. Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. *Ann Intern Med*. 1993; 119:771–778. [PubMed: 8379598]
15. Beutler B, Milsark IW, Cerami AC. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science*. 1985; 229:869–871. [PubMed: 3895437]
16. Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature*. 1987; 330:662–664. [PubMed: 3317066]
17. Reinhart K, Karzai W. Anti-tumor necrosis factor therapy in sepsis: Update on clinical trials and lessons learned. *Crit Care Med*. 2001; 29(7 Suppl):S121–S125. [PubMed: 11445746]
18. Lorente JA, Marshall JC. Neutralization of tumor necrosis factor in preclinical models of sepsis. *Shock*. 2005; 24 (Suppl 1):107–119. [PubMed: 16374382]
19. Ronco C, Bonello M, Bordoni V, Ricci Z, D'Intini V, Bellomo R, Levin NW. Extracorporeal therapies in non-renal disease: Treatment of sepsis and the peak concentration hypothesis. *Blood Purif*. 2004; 22:164–174. [PubMed: 14732825]

20. Zammattéo N, Girardeaux C, Delforge D, Pireaux J-J, Remacle J. Amination of polystyrene microwells: Application to the covalent grafting of DNA probes for hybridization assays. *Anal Biochem.* 1996; 236:85–94. [PubMed: 8619500]
21. Matteucci M, Caruthers M. Synthesis of deoxyoligonucleotides on a polymer support. *J Am Chem Soc.* 1981; 103:3185–3191.
22. Hermanson, GT.; Mallia, AK.; Smith, PK. *Immobilized Affinity Ligand Techniques.* San Diego: Academic Press; 1992. p. 454
23. Zhang Y, Singh VK, Yang VC. Poly-L-lysine amplification of protamine immobilization and heparin adsorption. *J Biomed Mater Res.* 1998; 42:182–187. [PubMed: 9773814]
24. Mansur HS, Orefice RL, Vasconcelos WL, Lobato ZP, Machado LJ. Biomaterial with chemically engineered surface for protein immobilization. *J Mater Sci Mater Med.* 2005; 16:333–340. [PubMed: 15803278]
25. Rutgeerts P, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, et al. Comparison of scheduled and episodic treatment strategies of infliximab in Crohn's disease. *Gastroenterology.* 2004; 126:402–413. [PubMed: 14762776]
26. Behm BW, Bickston SJ. Tumor necrosis factor-alpha antibody for maintenance of remission in Crohn's disease. *Cochrane Database Syst Rev.* 2008;CD006893. [PubMed: 18254120]
27. Doyle MK, Rahman MU, Han C, Han J, Giles J, Bingham CO III, Bathon J. Treatment with infliximab plus methotrexate improves anemia in patients with rheumatoid arthritis independent of improvement in other clinical outcome measures—A pooled analysis from three large, multicenter, double-blind, randomized clinical trials. *Semin Arthritis Rheum.* 2009; 39:123–131. [PubMed: 18823645]
28. Chow CC, Clermont G, Kumar R, Lagoa C, Tawadrous Z, Gallo D, Betten B, Bartels J, Constantine G, Fink MP, et al. The acute inflammatory response in diverse shock states. *Shock.* 2005; 24:74–84. [PubMed: 15988324]

FIGURE 1.

Bead modification chemistry: oxidation of the PSDVB portion of CytoSorb™ beads to incorporate carboxyl groups. Note that this schematic does not account for the presence of the PVP coating.

FIGURE 2. EDC activation chemistry: preparing carboxyl groups for covalent attachment to exposed amine groups on antibodies.

FIGURE 3. Experimental setup of cytokine capture experiments. The CAD is connected in line with a peristaltic pump and a reservoir of serum spiked with one or more cytokines.

FIGURE 4.

Anti-IgG-HRP surface density on beads containing adsorbed antibodies, beads containing covalently bound antibodies, and beads containing PLL-crosslinked antibodies. ($n = 3$ for each type of bead).

FIGURE 5.

TNF removal during 4 h recirculation using CADs packed with unmodified beads containing no antibodies (◆), beads containing covalently bound anti-TNF (○), and beads containing adsorbed anti-TNF (□). ($n = 3$ for each type of beads).

FIGURE 6.

TNF removal during 4-h recirculation using CADs packed with unmodified beads containing no antibody (◆), beads containing adsorbed anti-TNF (□), and beads containing adsorbed IgG (○). ($n = 3$ for each type of beads).

FIGURE 7.

Comparison of the simultaneous removal of IL-6 (a), IL-10 (b), and TNF (c) using unmodified beads containing no antibodies (◆) and beads containing adsorbed anti-TNF antibodies (□). ($n = 3$ for both types of beads).

FIGURE 8.

Rate of anti-IgG-HRP leaching (◆) and overall amount lost (□) during 2-h PBS/BSA flush of beads containing adsorbed antibody. This flushing step was performed prior to the cytokine capture shown in Figure 9. ($n = 3$).

FIGURE 9.

Comparison of TNF removal using beads containing adsorbed anti-TNF with ($\blacklozenge$, solid line) and without ($\square$, dashed line) 2-h preflush. The results of the flushing step performed on the beads used in these experiments can be seen in Figure 8. ($n = 3$ for both types of beads).