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This was the third Plasma Product Biotechnology meeting that I have had the pleasure to chair. PPB is establishing itself as the forum for everyone involved in the spectrum of activities making up the plasma industry to meet and discuss the latest challenges and developments in a relaxing atmosphere.

Challenges and developments were at the center of this year’s meeting. Held in the Caribbean, we heard about the challenges facing the countries of the region, such as getting blood tested for infectious agents, or even getting authorities to acknowledge that blood needs to be tested for such. This was sharply contrasted with the developments taking place in other parts of the world where sophisticated analytical techniques using mass spectrometry are in use, and methods are being developed to mine plasma for potential therapeutic proteins.

Since the first meeting in Australia in 1999, where the focus was more on plasma products, the program has evolved to incorporate issues within manufacturing, and more recently, developments within plasma proteomics. The four-day program was based on input from participants and regular visitors to our website. Building up the program in this way contributes to the success of the meeting as it focuses on topics that you feel matter most, while the relaxed atmosphere and informal setting promote discussion. This year’s program covered six main topic areas, Plasma Proteomics; Pathogen and Prion Safety; Developments and New Technologies; Regulatory Perspectives; Clinical Issues in Plasma Products; and Manufacturing Perspectives.

As after the previous meetings, we have put together a number of extended abstracts in the form of a small booklet; they are just a few of the many exciting and well delivered presentations given in Curaçao, Netherlands Antilles. After reading them, I hope you will be encouraged to join us next time in 2005.

I would like to thank all who participated, the Scientific Committee, my fellow organizers, and our co-sponsor CSL Ltd, Australia, for making this once again a successful Plasma Product Biotechnology meeting.

I look forward to seeing you all again in 2005.

Jan Berglöf
Chairman
Plasma products in retrospect

The plasma fractionation business, which began during World War II, has grown worldwide, evolving into a business with a significant and indispensable impact on human healthcare. The original small number of "early" products (albumin solutions, immune globulin and crude fibrinogen) has expanded over time with the advent of a variety of coagulation factor products and protease inhibitors. These products have not only been life-saving on countless occasions, but have also contributed to improving the quality of life of patients suffering from an array of diseases. Nevertheless, it should not be forgotten that in the past many of the very same products have inadvertently transmitted blood-borne infectious agents, thereby causing devastating diseases like hepatitis and AIDS in large numbers of the product's recipients. Fortunately, modern technological advances in plasma fractionation and present-day regulatory directives have reduced this risk to a very low level. For this reason, it can be stated that the plasma fractionation business has evolved into a mature industry, while still representing a relatively young field with a history of just over five decades. Nowadays the plasma fractionation industry is considered to be a traditional business and is even viewed by some as an old business. This is mainly due to the rapid evolution of biotechnology in the past 10–20 years which has resulted in the generation of a number of human plasma proteins produced by recombinant DNA technology.

Plasma-derived versus recombinant products

A number of biotechnological alternatives to the classic plasma-derived products are now produced in bioreactors, with some of them having obtained a marketing license, for example factor VIII, factor IX and activated factor VII. Products derived from transgenic sources are also underway, a few having entered the stage of clinical trials (e.g. antithrombin III (Edmunds et al., 1998), alpha-1-antitrypsin (Tebbutt S.J., 2000) and C1-esterase inhibitor) (Table 1).

Table 1. Sources and development stage of plasma protein products intended for clinical use.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Plasma-derived</th>
<th>Rec. DNA, cell culture</th>
<th>Rec. DNA, transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>++</td>
<td>+</td>
<td>o</td>
</tr>
<tr>
<td>Polyvalent immunoglobulin</td>
<td>++</td>
<td>–</td>
<td>o</td>
</tr>
<tr>
<td>Hyperimmune Ig's</td>
<td>++</td>
<td>+</td>
<td>o</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>++</td>
<td>+</td>
<td>o</td>
</tr>
<tr>
<td>Factor IX</td>
<td>++</td>
<td>+</td>
<td>o</td>
</tr>
<tr>
<td>Factor VII</td>
<td>++</td>
<td>o</td>
<td>–</td>
</tr>
<tr>
<td>Factor VIIa</td>
<td>(++)</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Thrombin</td>
<td>++</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>++</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Fibrin sealant</td>
<td>++</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Factor XI</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Factor XIII</td>
<td>++</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Von Willebrand factor</td>
<td>++</td>
<td>o</td>
<td>–</td>
</tr>
<tr>
<td>Prothrombin complex</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Activated PCC</td>
<td>++</td>
<td>o</td>
<td>–</td>
</tr>
<tr>
<td>Protein C</td>
<td>++</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Activated protein C</td>
<td>+</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Alpha-1-antitrypsin</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>++</td>
<td>o</td>
<td>+</td>
</tr>
<tr>
<td>C1-esterase inhibitor</td>
<td>++</td>
<td>o</td>
<td>+</td>
</tr>
<tr>
<td>Apotransferrin</td>
<td>+</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Mannan binding lectin</td>
<td>+</td>
<td>o</td>
<td>–</td>
</tr>
<tr>
<td>Apolipoprotein A-1</td>
<td>+</td>
<td>o</td>
<td>–</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>+</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Butyrylcholinesterase</td>
<td>o</td>
<td>o</td>
<td>–</td>
</tr>
<tr>
<td>Plasmin</td>
<td>o</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Synthocytes™</td>
<td>o</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VWF cleaving protease</td>
<td>–</td>
<td>o</td>
<td>–</td>
</tr>
</tbody>
</table>

++ licensed for clinical use
(++) not marketed anymore
+ in clinical trials
o in preclinical development, or recombinant DNA / transgenic expression achieved
– no product development known

At first sight one might think that the prospects for plasma products are waning, especially when one takes into account the initially alleged advantages of recombinant products: lower risk of transmitting infectious agents, unlimited supply, higher purity and low cost. However, it is now recognized that recombinant products cannot be considered to be totally free of the risk of transmitting infectious agents, at least as long as animal-derived ancillary materials are used during their production.
Secondly, the supply has not been flawless, as recent experience with e.g. recombinant factor VIII has shown. Thirdly, recombinant products are more expensive than their plasma-derived counterparts. Finally, the biotechnological production systems may generate protein variants differing slightly from the natural human ones, e.g. with respect to post-translational modifications. This may lead to a lower than normal in vivo recovery and half-life upon in vivo administration, as shown by recombinant factor IX in hemophilia B patients (White et al., 1997).

Production by recombinant techniques, especially those using mammalian cell culture systems, seems economically feasible only in the case of trace proteins. For proteins circulating in bulk, like albumin, it remains to be seen whether even transgenic production – with its potential for a high production capacity – will ever become financially competitive with plasma-derived proteins. For other products, we will have to wait and see whether it will be technically possible to produce an equally effective product by recombinant techniques, regardless of any financial-economic considerations. The best example of this is polyvalent immune globulin, which is increasingly used in a number of (auto-immune) diseases where it exerts an immune modulating effect. As long as the product’s active component is not elucidated in the clinical conditions concerned, it is unlikely that such a product will be mimicked by a recombinant alternative.

Taking all this into account it is clear that the classic plasma products will stay on the stage for quite some time to come. Some plasma derivatives will play a less prominent role, others will still show growth. With the proviso that plasma-derived products carry the lowest possible risk for transmitting infectious agents, competition based on product availability and price will mainly determine the market share taken by either type of product, plasma-derived or recombinant. Scientific and technological progress, combined with the impetus exerted by emerging recombinant products, has lead to a continuous improvement of existing plasma-derived products. These improvements relate above all to the clinical safety (lowering the risks of transmitting viruses and transmissible spongiform encephalopathies (TSE) agents; minimizing the occurrence of adverse events), as well as to increased efficacy, purity and process yields.

There is still a need for process steps dealing more effectively with non-enveloped viruses, while maintaining the protein yield at a high level. The ideal situation would be to combine virus reduction and high-yield product purification into a single process step to achieve the goal of low overall product loss. Fortunately, there are some promising leads underway, albeit still in an early phase.

New indications for already licensed products

Although a number of classic, plasma-derived products (factor VIII, albumin) are now used to a lesser extent, there are still opportunities for other plasma derivatives to expand their market potential.

Probably the best example is intravenous immune globulin (IVIG) for which the number of clinical indications is steadily increasing. Besides the now well-established indications of primary hypo- and agammaglobulinemia, secondary immune deficiency states, idiopathic thrombocytopenic purpura, and Kawasaki’s syndrome, recent additional indications include Guillain Barré syndrome and allogeneic bone marrow transplantation, while an increasing number of other diseases are the subject of clinical studies (prevention of graft-versus-host disease, certain auto-immune diseases, dermatomyositis, multiple sclerosis, and transplantation in general) (Kazatchkine and Kaveri, 2001). This off-label use of IVIG for treating a variety of (suspected) auto-immune diseases is now increasing in many countries, in particular for the treatment of neurological diseases. This has resulted in a steady growth in demand for this product, which is even causing shortages from time to time in some countries.

Another expanding market worth mentioning is that for fibrin glue or fibrin sealant. This two-component product, containing fibrinogen and thrombin, has been in clinical use in (mostly) Europe for some time, usually for providing local hemostasis during soft tissue surgery. Much attention, especially in the United States, is now being paid to the development of new applications for this product in a variety of clinical settings, mainly centering around a still increasing variety of surgical procedures and the promotion of wound healing (Morikawa, 2001). Fibrin glue has also been evaluated as a medium for controlled delivery
of drugs, such as growth factors and antibiotics (Jackson et al., 1996, Woolverton et al., 2001). Due to the product’s versatility, a number of companies are now developing, testing and registering several types of fibrin sealant.

C1-esterase inhibitor, a protease inhibitor less well known as a plasma product, also deserves attention. Its classic indication is treatment or prevention of attacks of edema formation, primarily in the oropharyngeal region and gastro-intestinal tract, in patients with hereditary angio-edema (HAE). Due to a deficiency (congenital or acquired) of this inhibitor, activation of the contact system may lead to uncontrolled generation of, eventually, bradykinin, which may result in life-threatening complications. The prevalence of HAE patients is low, which from a commercial point of view makes the C1-inhibitor product not very interesting to most plasma fractionators. At present, only three manufacturers produce this component from plasma, with at least one group working on a transgenic approach. However, other potential clinical uses of this protein based on animal models or proof-of-principle trials in humans have been described in the literature over the last 10 to 15 years. These include a.o. septicaemia, myocardial infarction, capillary leak syndrome, pancreatitis and organ transplantation, clinical conditions in which the anti-inflammatory properties of C1-inhibitor are exploited (Caliezi et al. 2000; Kirschfink and Mollness, 2001). In these conditions the product is not administered as a substitute, but rather as an agent inhibiting or attenuating the activation of complement and contact systems and thereby mitigating the inflammatory reaction, which has the potential to damage surrounding tissues. However promising the therapeutic potential of this plasma protein may be, it is clear that extensive, randomized, double-blind clinical studies are still required to prove the validity of this concept.

Alpha-1-antitrypsin (AAT) derived from human plasma has been on the market for almost 15 years for treating pulmonary emphysema caused by congenital AAT deficiency. However, this protease inhibitor is also believed to be of therapeutic value in cystic fibrosis and certain skin diseases like dermatitis and psoriasis (Anonymous (a), Anonymous (b)). With these large potential markets it is not surprising that there are other companies, in addition to plasma product manufacturers, showing interest in this protein with attempts to develop a recombinant alternative.

Until the advent of high-purity factor IX concentrates, prothrombin complex concentrates were routinely used in the treatment of hemophilia B. In some countries today the product is still used for counteracting the effects of cumarin overdose. A potential indication is severe liver insufficiency, resulting in low levels of vitamin K-dependent coagulation factors. Naturally, this application necessitates that the product does not exert thrombogenic activity.

Options for new therapeutic components

At present, the range of plasma derivatives in clinical use may seem pretty large, comprising roughly twenty different proteins (Table 1), but it should be realized that plasma contains thousands of proteins, the functions of which are still unknown in most cases. One modern approach to explore this protein mine is to use high-throughput systems. Here proteins are isolated using ligands based on combinatorial chemistry and subjected to screening systems designed to measure a desired biological activity (Lathrop et al. 2003). However, the conventional approach of purifying proteins one by one and looking for their function is also still effective: ideas abound for clinical applications for a number of plasma proteins which at present are discarded as waste material in routine plasma fractionation schemes. Which compounds might we consider? The following is an outline of some opportunities that are presently being evaluated.

A candidate receiving much attention is mannan-binding lectin (MBL), a component of the so-called innate (aspecific) immune system. This protein, present in plasma in association with two serine proteases (MASP-1 and MASP-2), has a high affinity for repetitive structures of mannan groups, like those occurring on the surface of (pathogenic) micro-organisms. After binding to these structures, the MBL/MASP-1/2 complex will activate the complement system, which leads to the destruction of a large variety of micro-organisms. A congenital deficiency of MBL may lead to recurrent infections, especially at an early age when the specific immune system has not yet matured. As congenital deficiencies of MBL are relatively
frequent in the normal population, this opens the possibility to apply MBL to treat MBL-deficient patients having a primary or secondary immune deficiency who thus risk contracting infections (neonates, children with leukemia, patients with acquired immune deficiencies or certain viral diseases, etc.). Cystic fibrosis, rheumatoid arthritis and recurrent miscarriage may also be targets for MBL therapy (Kilpatrick, 2002(a); Kilpatrick, 2002(b)). An experimental product has already been applied clinically in a setting of compassionate use (Valdimarsson et al. 1998; Garred et al. 2002), but well-designed clinical trials still have to reveal the real therapeutic value of this protein.

Apotransferrin is another example of a product opportunity. This iron transporting protein may be utilized in its iron-free form as a means to prevent cytotoxic effects exerted by accumulating free non-transferrin bound iron (Fe^{3+}) in conditions where normal iron utilization is hampered and/or apotransferrin production is decreased. This is the case in leukemia patients after high-dose chemotherapy or after stem cell transplantation. A stable, viral-safe, clinical-grade product has recently been developed starting from Cohn fraction IV and is now in clinical trials (Von Bonsdorff et al. 2001, Sahlstedt et al. 2002).

For butyrylcholinesterase, potential indications are cocaine overdose, apnoea following anesthesia of patients deficient in butyrylcholinesterase, and treatment or prevention of nerve gas intoxication (Mattes et al. 1997, Browne et al. 1998, Ralston et al. 2002). This product too can be isolated from Cohn fraction IV.

Fibronectin was reported recently as a candidate for treating chronic skin wounds caused by poor circulation in the lower limbs, such as diabetic foot ulcers and venous stasis ulcers, due to the fact that the protein is involved in most processes of wound healing. Clinical trials with fibronectin dealing with wound healing have shown variable results in the past, but the protein is now going to be tested in new clinical studies (Horowitz and Beaulieu, 2002).

Plasmin is currently being tested in animal models with the intention of using it as a new fibrinolytic agent in humans, to be applied directly to the clot through a catheter in cases of peripheral arterial occlusion. In animal studies this approach compares favorably with licensed fibrinolytic agents like tissue plasminogen activator, particularly when blood clots have been formed for hours and no longer contain a great deal of activatable plasminogen (Novokhatny and Petteway, 2002; Marder et al. 2001).

Apolipoprotein A1 has been purified from Cohn fraction IV and from precipitate B of the Kistler and Nitschmann ethanol fractionation process with effective virus reduction steps. Following recombination with lecithin to form "reconstituted high-density lipoprotein" (rHDL), this compound may be applicable as an anti-inflammatory therapeutic in septic conditions since it is able to neutralize endotoxin and thereby prevent excessive release of cytokines in vivo (Levine et al. 1993; Hubsch et al. 1993; Lerch et al. 1996; Pajkrt et al. 1996). This rHDL preparation was also shown to have an inhibiting effect in vivo on endotoxin-induced activation of coagulation and fibrinolysis and ex vivo on collagen-induced platelet aggregation (Pajkrt et al. 1997). To date, the beneficial effect of this product in the treatment of sepsis has not been studied in full-scale clinical trials. Current expectations are that rHDL will be of benefit in cardiovascular disease due to its protective effect on the vascular wall. The product's inhibitory properties on endothelial cell activation and its effect on reverse cholesterol transport may be of benefit in hypercholesterolemic patients and in atherosclerosis (Spieker et al. 2002; Lerch 2002).

Much research is presently being conducted on Von Willebrand factor cleaving protease (VWF-CP, ADAMTS-13). The physiological function of this enzyme appears to be the cleaving of ultra-large multimers of Von Willebrand factor as they enter the blood stream directly after biosynthesis by endothelial cells (Dong et al. 2002). These ultra-large multimers promote platelet aggregation under high shear stress conditions, such as those present in the microcirculation, thereby leading to the syndrome of thrombotic thrombocytopenic purpura (TTP) (Furlan and Lammle 2001; Moake 2002). Although the VWF-CP level is never zero in TTP patients, it is clearly decreased. This may be due to a congenital deficiency, as is the case in children with chronic relapsing TTP, or to idiopathic acquired VWF-CP deficiency associated with certain auto-immune diseases or drug-induced TTP. TTP patients are now treated by infusing plasma as a
source of VWF-CP, but a more effective and safer therapy will be possible when a specific, purified VWF-CP product is made available.

Human albumin microspheres coated with fibrinogen have been developed as a substitute for human platelets (Synthocytes™) (Heath et al. 1999). In a thrombocytopenic rabbit model it was possible to demonstrate their efficacy for arresting bleeding from surgical wounds as well as a correcting effect on the prolonged bleeding time (Levi et al. 1999). As assessed in a perfusion chamber coated with human endothelial cell matrix, the mode of action seems to rely on the interaction of the beads with circulating platelets, facilitating platelet adhesion to the endothelial matrix (Levi et al. 1999). In in vitro studies it was demonstrated that Synthocytes interacted directly with platelets, probably through interaction of the fibrinogen layer with GPIIb/IIIa on the platelet surface, which led to activation of the platelets or increased susceptibility to physiologic platelet activation agents (Davies et al. 2002). This innovative product could be of particular use in thrombocytopenic patients who have become refractory to infusions with normal platelet concentrates due to allo-immunization.

Prospects and opportunities for plasma-derived products

Although the survey given above is by no means exhaustive, it demonstrates that there is still a promising future for plasma derivatives. That is not to say that new markets will easily be established and that blockbusters like (formerly) albumin and factor VIII are on the horizon. Some product opportunities have the potential to achieve that status (C1-esterase inhibitor, alpha-1-antitrypsin), other products may only serve niche markets, which on a world-wide basis can still be commercially interesting. Serving a niche market also opens the possibility of applying for an orphan drug status, a route which is increasingly followed by many manufacturers. To obtain this status the prevalence of the disease concerned should be less than 1:2,000 for Europe and Australia, (in principle) 7.5:10,000 for the United States, and a total afflicted patient number of less than 50,000 for Japan.

In all these cases appropriate clinical studies still have to show whether the expectations are justified. These clinical trials, in combination with the subsequent marketing licensing procedures, require both considerable effort and substantial investments, something which the smaller players in the field will find difficult or even impossible to afford. It is to be hoped however, that even these compounds will be tested, and, if shown to be clinically effective, brought to the market for the benefit of patients.

Once a new plasma protein has shown its merits in clinical use, recombinant alternatives may follow. However, a recombinant route is not always successful. Due to the long development times, the remaining validity of protecting patents may have become very short by the time the corresponding recombinant product gets its marketing license. But even when a recombinant alternative is successful, it does not automatically mean that the plasma-derived counterpart will succumb to it, provided that the quality and clinical safety are at least comparable to those of the recombinant product.

A major challenge for both the plasma fractionation and the biotechnology business is to provide high-quality therapeutic proteins which are affordable for patients living in countries less rich than those of the western world. A typical example of this challenge is the treatment of patients suffering from hemophilia: both plasma-derived and bio-engineered factor VIII and factor IX products are too costly to serve the hemophiliac population in developing countries, and it is not expected that this situation will change in the near future. Transgenic products have the potential to be cheaper, but these still have a long development ahead. The plasma industry has an economic advantage over the biotech industry in that several components can be recovered from one source: the multi-component reservoir that plasma actually is. This plasma can be collected by both developed and developing countries. Economy of scale is the factor determining whether the fractionation of this source material is to be carried out nationally, or contracted out to a large facility abroad. However, the advantage of plasma as one source material is in itself not sufficient to guarantee the provision of low-cost products. There is an equally large need for
cost-effective and high-yield processing methods, including those required for guaranteeing viral and prion safety. Finding a solution to the problem of making available state-of-the-art, high quality and affordable plasma derivatives presents an ethical issue of enormous importance.

In conclusion: the plasma business should definitely not be regarded as old-fashioned, taking into account its prospects, which are based on the current progress in science and technology, a relatively high flexibility, and the use of high quality standards.

References
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Kilpatrick, D.C. Mannan-binding lectin and its role in innate immunity. Transfus. Med. 12, 335–352 (2002 (b)).


Safety of blood and blood components for transfusion in Latin America and the Caribbean

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The Pan American Health Organization (PAHO) receives information on a yearly basis from the countries and territories of Latin America and the Caribbean on blood collection, screening for infectious agents, and separation into components. The data presented here are a summary of the official information provided to PAHO for 2002, in comparison with figures obtained for the previous three calendar years.

During 2002, 7 824 291 units of blood were collected in the 40 countries and territories from which data were received. This number of units represent a donation rate of 14.7 units of blood per 1 000 inhabitants, an increase of 29% over the rate for 1999, 11.4 per 1 000.

Table 1. Units of blood collected in Latin America and the Caribbean, and Donation Rate per 1 000 inhabitants.

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of units</th>
<th>Donation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>5 996 591</td>
<td>11.4</td>
</tr>
<tr>
<td>2000</td>
<td>6 390 242</td>
<td>12.1</td>
</tr>
<tr>
<td>2001</td>
<td>6 831 001</td>
<td>12.8</td>
</tr>
<tr>
<td>2002</td>
<td>7 824 291</td>
<td>14.7</td>
</tr>
</tbody>
</table>

The donation rate is low in most countries of the Region. Only Aruba (35.0), Uruguay (35.0), Curacao (40.7) and Cuba (53.8) have rates above 20 per 1 000 inhabitants. Most of the remaining countries have donation rates below 10 per 1 000 inhabitants. For comparison, the rates for Canada and the United States of America are 32.7 and 45.9, respectively (Fig 1).

The vast majority of the blood units is collected from replacement donors. Replacement donors are individuals, either relatives or friends of patients, who are required to give blood in response to specific requests from the health providers. Aruba, Cuba and Curacao report 100% voluntary blood donation; British Virgin Islands, Cayman Islands and Bermuda have over 90% voluntary donors, while Bolivia (4%), Honduras (9%), Panama (47.9%) and Peru (3%) report remunerated donation.

There is a tendency for higher prevalence rates for antibodies against human immunodeficiency virus (HIV) and hepatitis C virus (HCV), and of hepatitis B surface antigen (HbsAg) among donors in the countries where voluntary blood donations...
is either inexistent or low. The higher prevalence rates are observed in countries where individuals received remuneration when giving blood for transfusion (Fig 2, Table 2).

Table 2. Prevalence of transfusion-transmitted infections among blood donors in Latin America and the Caribbean.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>With &gt; 98% voluntary</td>
<td>0.09</td>
<td>0.002</td>
<td>0.22</td>
<td>0.15</td>
</tr>
<tr>
<td>blood donors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With paid donors</td>
<td>0.28</td>
<td>0.35</td>
<td>0.52</td>
<td>0.58</td>
</tr>
<tr>
<td>All other countries</td>
<td>0.18</td>
<td>0.34</td>
<td>0.38</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Screening for infectious agents is not universal in the Region. The consensus is to test all units for HIV, HCV, HBsAg and syphilis in every country, and for T. cruzi in the continental Latin American countries. However, of the eligible units, 0.14%, 0.19%, 0.56%, 0.10% and 6.16% respectively, were not screened for the above mentioned markers. This is associated primarily with the lack of a permanent stock of blood in the blood banks, resulting from replacement donation in some countries and, to a lesser extent, with the absence of specific screening reagents. In some of the latter cases, the health authorities have decided not to procure the laboratory reagents because they consider that the prevalence of hepatitis C and T. cruzi infection is low in their countries and, therefore, the screening of donated blood is not warranted.

Screening of blood prevents the transmission of infections only if the tests results represent the true infection status of the donors. False negative results can be associated with the window period, with lack of sensitivity of the testing kits, and with the inability of the testing center to carry out the tests appropriately. Programs for external evaluation of performance may provide retrospective information on the accuracy of the testing results. PAHO’s regional program on external evaluation of performance, in which at least one of the major national blood banks participates, shows that negative results are reported. Furthermore, the likelihood of providing both false negative and false positive results is higher among centers that handle fewer samples (Fig 3).
The region containing China and South East Asia is one of the fastest growing economic zones in the world, with many countries experiencing double digit growth in recent years. This growth has been fuelled by a collective population in excess of 1.7 billion, presenting highly competitive labor costs and attracting investment in a wide range of manufacturing activities from steel through pharmaceuticals. This rapid economic growth has created a burgeoning affluence in sections of this population and enhanced demands for improved housing, education and healthcare. This presentation addresses the impact of the rapidly changing environment on one aspect of the health care system, namely the plasma fractionation industry, and focuses largely on China, the only country within the region to operate its own plasma fractionation facilities.

China has a population of approximately 1.3 billion but approximately 70% of the population live in rural areas and access only about 20% of the total healthcare budget. The expenditure on healthcare represents around 4% of the Chinese GDP, compared to 14% in the USA, but this figure has been growing at a rate of 16% per annum over the last decade, driven mainly by an ageing population, higher living standards and enhanced health awareness. Despite the significant improvement in the overall healthcare in the country, there remains a general view, however, that government monitoring and acknowledgement of the incidence and treatment of specific diseases such as human immunodeficiency virus/acquired immune deficiency syndrome (HIV)/AIDS and severe acute respiratory syndrome (SARS) remains poor.

Plasma collection in China is controlled by a network of about 200 state- or local government-owned centers that collect a total of around 4 million liters of plasma each year. The Ministry of Health allocates to each fractionator the exclusive rights to the output of collection centers from 3 separate provinces and in return the fractionators assume the responsibility for the management of the centers and their compliance to government regulations. The cost of plasma to the fractionators in China is US $40/liter and the export of plasma is illegal.

The plasma fractionation industry in China has historically utilized a network of state-controlled Cohn fractionation facilities operated by the China National Blood Products Corporation, the local provincial governments, or the Peoples Liberation Army. The majority of these plants were of low operational capacity (less than 100,000 liters per annum) and of poor design, well below the GMP standards required of western plasma fractionation facilities. The one exception to this is Shanghai RAAS, the modern Sino-American joint venture facility located on the outskirts of Shanghai, with a reported operational capacity of 2 million liters. This facility was established in 1988 and produces a wide range of plasma products by Cohn technology. By the mid 1990s there were as many as 60 plasma fractionation facilities operating in China, but this picture has changed dramatically in recent years (see Table 1). The relaxation of central government control and the opening of state run facilities to entrepreneurial capital and management practices has created a more competitive environment, causing many of the smaller, inefficient provincial government facilities to cease production. Currently there are 34 plasma fractionation facilities operating in China, but predictions are that this number could fall to as low as 16 fractionators by 2005. In addition, central government initiatives aimed at a) improving the quality of blood collection procedures, b) rigorously enforcing compliance to the code of GMP introduced into China in 1998, and c) encouraging the construction of new plasma fractionation facilities and modernizing existing facilities with new technologies are further likely to enhance the consolidation already evident in the industry. Figure 1 summarizes the key events that have occurred in the Chinese plasma fractionation industry over the last 15 years.
The total market for all plasma products in China in 2000 was US $298 million, which represents a 3-fold increase in six years (Table 2). The range of products manufactured is however very limited with albumin sales dominating the market. Current figures indicate that approximately 100 tonnes of albumin were produced in China in 2001 and this represented over 60% of the total plasma product sales. Limited reimbursement is available for albumin used for specific indications and foreign products may be imported but are subject to a 10% importation tax and a 17% VAT.

The intravenous immune globulin (IVIG) market is dominated by the Chengdu Institute of Biological Products which supplies 50% of the market while Shanghai RAAS holds 27%. Three tonnes were produced in 2001 and the importation of foreign product is prohibited. There is no health cost reimbursement for albumin used for specific indications and foreign products may be imported but are subject to a 10% importation tax and a 17% VAT.

The factor VIII market is small. Only 30–35 million International Units (IU) were administered throughout China in 2001, a treatment level 100 fold lower than in the West and consistent with the view that the market is substantially under-diagnosed. Importation of foreign product is prohibited.

China’s entry into the World Trade Organization (WTO) will introduce further changes in the plasma products market. The reduction in the 10% tariff on imported plasma, the acceptance of other imported plasma products, including IVIG and factor VIII into the marketplace, and enhanced price transparency will increase the quality of product available. At the same time the movement, already underway, towards private healthcare systems in China will fund the growth in plasma products in both quantity and diversity.

Outside of China, there are no plasma fractionation facilities in the South East Asian region. CSL in Australia toll fractionates for Red Cross Organizations in Hong Kong, Malaysia, Singapore and New Zealand, while the Scottish National Blood Transfusion Service (SNBTS) fractionates some Taiwanese plasma. There appear to be no plans for the construction of a new plasma fractionation facility in any South East Asian country in the near future and commercial product is readily available, although subject to considerable price volatility.

In conclusion, the plasma fractionation industry in China and South East Asia is currently in a poorly developed state. No South East Asian country operates a plasma fractionation facility and these nations rely on product supplied from the large commercial fractionators. In China, the fractionation plants are for the most part well below the regulatory compliance standards expected in the West but the relaxation of central control by the Chinese Government and the burgeoning Chinese economy is having a dramatic influence on the industry. A number of companies are pursuing initiatives aimed at introducing modern fractionation protocols and in the next few years both the quality and range of plasma products produced by these new or revamped Chinese plants is likely to increase dramatically. This movement, along with Government sponsored initiatives directed at improving plasma collection procedures, and the ready availability of comparatively inexpensive Chinese plasma is likely to translate into China becoming a net exporter of quality plasma products for the South East Asian region and developing nations around the world.
Within human plasma are millions of proteins, including antibodies, protein complexes, protein fragments and proteins with alternative post-translational modifications or products of alternatively spliced mRNA, many of which may have therapeutic implications; however, efforts to exploit this rich potential have been slow to realize success. Attempts to address this deficiency are being made on many fronts. For example, the Human Proteome Organization (HUPO) seeks to identify every protein in plasma. Over 40 international groups are using several different methods, many based on mass spectrometry to achieve this goal. While over 1000 proteins in plasma have been identified, current methods are hampered by differences in the abundance of plasma proteins, which are estimated to cover ten orders of magnitude (1). Therefore, detection of rare proteins requires removal of the most abundant plasma proteins including albumin and the immunoglobulins. This removal clearly limits the new therapeutic activities that can be detected in plasma as, for example, antibodies possess profound physiological activities. Furthermore, the overwhelming amounts of data generated in proteomic catalogs do not provide functional indications of which proteins will best serve as targets or new therapeutics.

The American Red Cross has developed a discovery methodology that has the ability to discover desirable biological activities, including therapeutic drugs themselves or targets for therapeutic intervention. The basis of this discovery lies in the ability to separate and concentrate most, if not all, of the proteins in a highly complex material, based on their unique conformation; and to simultaneously evaluate all of the components for a desired biological activity. The components include functional protein complexes, different conformations and post-translational modifications, proteolytic fragments, and antibodies from the human immune repertoire. This discovery is unbiased in regard to the functional entity that is discovered and requires no foreknowledge of its structure or identity: all elements of the starting material are present in the assays and thus have an equal opportunity to be selected if they possess the desired function.

To begin, a solid phase combinatorial library of small molecular weight ligands is synthesized by "split-couple-recombine" methods (2,3) on chromatography resin beads. Each bead has millions of copies of a single ligand that is different from the ligand on every other bead, such that a library may comprise millions of different ligand structures. The ligand library is then incubated with complex source material e.g. pooled human plasma, disease-state specific plasma, etc. Protein will naturally bind to the ligands by means of the specific interactions of the target molecule in the plasma with the ligand, and will become concentrated on the beads due to the relative affinity of the target for the ligand. Non-bound protein is removed by washing, and the binding and washing conditions may be designed to increase a certain type of binding, e.g. ionic or hydrophobic interactions. The bound protein-ligand complexes are then simultaneously evaluated for activity in a relevant biological assay e.g. cell growth, cell death, migration, differentiation etc. In the course of the assay, bound protein will slowly elute from the bead due to the inherent dissociation rate of the ligand-protein interaction. The effect of the dissociated protein in the assay may be viewed through microscopy, and powerful data handling capabilities will enable precise identification of active bead. The bead associated with the activity is collected, and its coupled ligand sequenced by standard Edman degradation or peptide sequencing methods. A preparative scale chromatography medium bearing the ligand is synthesized and the active entity purified from the column based on the identification of active fractions. The protein can be subsequently identified using standard and state-of-the-art analytical means.
A number of proof-of-principle studies demonstrate the feasibility of this technology. In a first example, identification of factors that will support cell growth was demonstrated. New treatments for promoting cell growth are needed to treat indications such as improving the healing of chronic wounds. In a model, the ligand bead was made by binding an anti-IL-2 antibody to Protein G Sepharose™ beads (Amersham Biosciences). These beads were incubated with recombinant human IL-2 spiked into plasma. The beads were then co-cultured with human NK-92 cells, which require the addition of IL-2 to the medium in order to grow; however, no exogenous IL-2 was added to the cells. Growth of cells was indicated by growth in healthy clumps, whereas death was determined by uptake of the red fluorescent dye propidium iodide (PI) using the detection technology of Automated Cell Inc. Growth was observed only in wells in which the beads were incubated with IL-2 and quantitated (Fig 1).

In a second example, we have demonstrated that disease-related antibody may be identified using our technology. Disease-related antibodies are a factor in autoimmune diseases including systemic lupus erythematosus and multiple sclerosis. Identification of these antibodies can help in diagnosis or in development of a target for therapeutic intervention. To demonstrate the feasibility of this method in autoimmune disease, a cohort of mice was immunized with ovalbumin and the IgGs from these and control mice were purified. The IgGs from the immunized mice were labeled with rhodamine and the IgGs from the control mice were labeled with FITC. The labeled antibodies were mixed and incubated with a library of hexamer ligands. The antibodies from the two populations were fractionated on the ligand library according to their various affinities. The majority that bound antibody was both red and green, indicating that they bound antibody from both populations. About 1:20,000 beads fluoresced only red, indicating that they bound antibody only from the immunized population. This method can be used to identify antibodies that are involved in autoimmune disease as both biomarkers or as targets of potential therapeutics.

Additional disease-related bioassays include screening for antibodies that cause death of cancer cells, peptide fragments that act as anti-infectives, and diagnostic biomarkers. Current discovery methods in proteomics generally seek to identify all of the proteins found in plasma, or to identify function from a defined sub-proteome. The discovery method described above is unbiased, in that all of the proteins in plasma are included in the assay and are tested for a desired function, but only those that have the desired activity are developed further. This technology should facilitate the discovery of previously unknown proteins, further expanding the uses, and developing the riches contained in the depths of plasma.

Reference

Fig 1. Growth of cells incubated with anti-IL-2 beads bound with IL-2. Experiment was conducted as described in the text. A. Death of cells indicated by PI uptake. B. Growth of cells indicated by growth of clusters of cells.
According to its cell tropism, parvovirus B19 (B19) is classified as genus Erythrovirus in the virus family parvoviridae. The virus has a double stranded DNA, size 5.6 kb, and no virus envelope. It replicates in erytroid progenitor cells (bone marrow, peripheral blood, fetal liver). The virus is present worldwide and in Europe about 60% of the middle-aged population has antibodies against this virus. The virus carries some risk for immunocompromised patients and for pregnant women.

Transmission of B19 via plasma products had been observed in rare cases. It was demonstrated that hemophilia A patients acquire the infection earlier than the normal population and are 100% antibody positive at a relatively young age (1). This drew attention to the fact that B19 may be transmitted by factor VIII concentrates.

From 1999 to 2000 several plasma pools were tested for B19-DNA contamination (2). The findings of previous publications were confirmed: B19-DNA was present in 222 of 372 pools (60%) and the level of contamination in some batches was \( \geq 10^7 \) geq/ml. B19-DNA was also found in plasma derivatives: 79 of 91 batches of factor VIII concentrates (87%) contained B19-DNA and the level of contamination varied between \( \leq 10^3 \) and \( \geq 10^7 \) geq/ml. Even factor IX concentrates were contaminated with B19-DNA (41 of 62 batches, 66% positive). Although the level of contamination in these cases was generally lower, 18% of the batches had contamination levels of about \( 10^6 \) geq/ml. In contrast, immunoglobulins, albumin and AT III preparates were seldom contaminated and if B19-DNA was found, the level of contamination was low, often at the detection limit of the assay (\( \leq 10^3 \) geq/ml).

Because of the restrictive cell tropism of B19 and that there is no cell system available for routine use, animal parvoviruses, (porcine (PPV), canine (CPV), bovine (BPV), and Minute Virus of Mice (MVM)) have been used for studying the effectiveness of virus removal or inactivation procedures with respect to parvoviruses. It has been shown that animal parvoviruses are not inactivated by heat treatment like pasteurization, a required treatment for albumin (European Pharmacopoeia) and other plasma derivatives. With the availability of the erythroid leukemia cell line, the KU12EP6 clone (3), it became possible to investigate the inactivation of B19 in albumin by pasteurization. It was demonstrated, that in contrast to animal parvoviruses, the human B19 was heat sensitive and could be completely inactivated. In order to clarify this effect, the inactivation kinetics were demonstrated at reduced temperatures (53, 54, 55.5 and 60 °C). These data clearly showed that a reduction in temperature delayed the inactivation and the treatment was ineffective at 53 °C. The complete inactivation of B19 is only informative for albumin, which is pasteurized in the final container. That B19 is more heat sensitive than the animal parvoviruses is only demonstrated for albumin; more investigation with regard to other treatments and products is needed.

B19 transmission via coagulation factor concentrates that have undergone virus inactivation heat treatment has been observed. Two transmission cases were characterized by comparing the genome sequences isolated from the patient and from the product (4). Both cases were detected by seroconversion of the patients and not by clinical symptoms. These two cases demonstrate that heat treatment alone is not effective enough to inactivate B19 and thus prevent its transmission, even though in one case the product was contaminated with B19 to a low level (\( 4 \times 10^3 \) geq/ml). This draws the attention to the fact that hemophilic patients can receive high dose concentrates which may result in the accumulation of B19 in the patient.
In summary, the data available at present suggest that there are differences between the properties of animal and human parvovirus. The difference in heat sensitivity has been demonstrated in albumin. These results do not allow the conclusion that B19 is in general heat sensitive. As demonstrated by the B19 transmission cases, knowledge of the properties of B19 in relation to the different heat treatments applied today for production of plasma derivatives is insufficient. Further investigation is needed.

References

Polyclonal IgG is the highest selling human plasma-derived product and continues to drive the plasma industry. Sales in 2001 were approximately US $2 billion, growing at 2% per year; consumption was estimated at 50 metric tons, growing at 6% per year (1). Previous contamination of IgG by enveloped viruses such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV) raised concerns over product safety. Today contamination by enveloped virus has been addressed through the adoption of virus clearance technologies such as Solvent Detergent, heat, low pH, caprylate, chromatography, and large virus filtration. Current safety concerns are focused on the smaller, non-enveloped viruses such as hepatitis A virus (HAV), parvovirus B19, and new agents like transmissible spongiform encephalopathies (TSE), West Nile virus, (WNV) and severe acute respiratory syndrome (SARS).

The use of virus filtration for non-enveloped virus clearance has a number of benefits. Filters are inert and do not degrade the product or add any foreign substances that later require removal. They are also capable of high yield, can be validated using scale-down models and in-process integrity tests, and can be conveniently implemented into a manufacturing process. The Millipore Viresolve™ NFP is a normal flow parvovirus removal filter with a patented composite membrane structure. It retains > 4 log 10 reduction value (LRV) of non-enveloped, 20 nm diameter paroviridae with high flows (75–150 liters/m²/h (LMH) at 30 psi) and high capacities (100–200 liters per 10 inch cartridge).

The development of a parovirus filter process requires examining how the independent, or design variables (for example, feedstock and operating conditions) affect the dependent, or performance parameters (for example, sizing, product recovery, and LRV). Virus filter sizing is determined by flux (J, LMH) and capacity (V_max, l/m²), as shown in the formula. As shown in the figure, a high flux (filter b) is more effective at moderate process times than a high capacity filter. NFP filters, being high flux filters, thus offer manufacturing flexibility, such as operation in line with another process step. Other non-enveloped virus filters with low flux and high capacities are economical at extended process times.

\[
\frac{m^2}{L} = \frac{1}{V_{max}} + \frac{1}{J \cdot t}
\]

Average flux (sizing) is proportional to the applied pressure. For clean streams, the flow decay is primarily due to osmotic pressure effects from the high concentration at the membrane surface. In these instances, increasing pressure can improve filtration throughput; 50 psig is typically the optimum operating point. Capacity can decline with protein concentration, suggesting the existence of an optimal protein concentration that minimizes virus filter area requirements (US Patent 5,597,486). This concentration for IgG is in the range of 6–10 g/l.

All normal flow virus filters show a decline in LRV with volume processed (2). This decline varies with different feedstocks and can be conveniently correlated with the extent of flow decay for the Viresolve NFP filter. A model plugging agent, 2000 kDa Blue Dextran, is used to demonstrate this phenomenon with bacteriophage Phi-X 174. Varying the concentration of Blue Dextran gives 4 LRV endpoints over a throughput range from 10 to 100 l/m². When these data are correlated to flow decay, the 4 LRV endpoints are all at roughly
80% flow decay. By restricting the flow decay to less than 75%, a 4 LRV of virus clearance can be maintained. High retention and economical sizing are both obtained by increasing capacity and reducing filter plugging.

Virus filter capacity is affected by aggregates in the feedstock. Model aggregates created by heating IgG (sized at 10^7 Da by light scattering) plug the virus filter at mg/l concentrations. The nature and amount of aggregates depend on the protein, its purity and source, buffer conditions, hold time, and temperature. Optimization of buffer conditions such as pH and ionic strength can minimize area requirements for NFP filtration. Implementation of NFP filtration before a diafiltration step allows optimization of the buffer conditions and protein concentration for virus filtration; any unwanted buffer components can then be diafiltered away in the next step. Freezing of protein solutions can often introduce an aggregate level which will impact NFP capacity.

Prefilters can be used to remove aggregates and increase the capacity of the virus filter. A disposable NFF prefilter using cellulose fibers with high purity diatomaceous earth can be used downstream in the process to improve virus filter capacity (patent applied for). This filter has lower extractables than current lenticular-pad based filters and will be appropriate for use in high purity downstream applications. The NFF prefilter removes aggregates by an adsorptive mechanism. Typical prefilter capacities are 200–400 l/m² and can improve the NFP capacity five-fold.

A reusable TFF prefilter using a composite regenerated cellulose (Ultracel™) ultrafiltration membrane with a 2000 KD nominal molecular weight cutoff retains aggregates and passes IgG monomer/dimer (US Patent 6,365,395). This membrane is employed in a cassette format with low pressure drop feed channels and is operated in TFF mode with permeate flux control. Utilization of this prefiltration technology can improve the NFP capacity dramatically, as much as tenfold. Depending on the number of kg of IgG processed by the TFF system annually, the overall filtration costs can range from US $0.75–2.00/g.

Parvovirus removal by filtration using Viresolve NFP is a viable clearance process with capabilities of >4 LRV of parvovirus, yields of >97%, and economics of US $1–3/gm of IgG. Optimization variables include protein concentration, buffer and pH, and prefiltration. High aggregate levels limit the capacity of virus filters so that either a disposable NFF prefilter or a reusable TFF prefilter can be used to extend virus filter capacity for economical sizing and a robust process.

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References
Viral safety in biopharmaceuticals is a subject of continued concern. Currently all biotechnologically produced pharmaceutical products, including the products from plasma fractionation, are subject to the strict demands and regulations imposed upon all pharmaceutical agents that are directly administered to an animal or a human. In addition to the efficacy of the products, manufacturers also have to demonstrate their safety. Quantitative data concerning respective manufacturing steps or defined antiviral methods are obligatory. The necessity of more than one virus clearance step in the same production process is indispensable today, since not only known but also unknown, unsuspected, and harmful viruses with different biophysical and structural qualities have to be inactivated or removed. Even if the technologies available for eliminating small, non-enveloped viruses for many biofluids are limited in comparison to the control of large, enveloped viruses, there are some promising new approaches using ultra short term high temperature or ultra violet (UV) irradiation devices. Inactivation of viruses is achieved by the destruction of their lipid or protein covers, making them unrecognizable to the target cell, or by destroying the viral nucleic acid, and therefore the biological activity necessary for replication.

Irradiation of biological and other fluids with UV light has been employed as a method for inactivating undesirable micro-organisms. UV-treatment of blood derivatives is especially useful for treating uncoated, heat-stable viruses. An inherent problem in the application of UV-irradiation techniques is controlling the irradiation of a fluid to ensure sufficient exposure to inactivate microorganisms,
whilst minimizing or avoiding damage to desirable proteins and other components within the fluid. Attempts to address these limitations have led to the development of a new type of reactor. The fluid moves in a primary flow directed along the length of a UV lamp, and a secondary circulating flow is induced within the fluid. The secondary flow is superimposed on the primary flow, providing uniform and controllable exposure of the entire volume of fluid to UV radiation.

Through laboratory experiments, process parameters have been defined for scale-up to industrial processes. Fouling problems, coupled with the special demands on biotechnological systems for cleaning and validation requirements, determine the construction of such apparatus. Recently conducted experiments with model viruses containing protein solutions highlighted the critical parameters when accessing UV sterilization methodologies.

Our efforts led to virus inactivation and desinfection systems for UV-C treatment of biotechnological or human blood plasma-derived products with a brand new reactor type where the treatment chamber is formed by a tubular PTFE conduit which spirals around a quartz tubing with a concentric UV-C source (254 nm). The units are expected to be the most efficient, safest and cost-effective treatment systems on the market and can also be used in food and beverage industries and other liquid applications. As a superior alternative or supplement to common methods of virus reduction like heat, chemical desinfection, chromatography or filtration, the systems even inactivate non-enveloped viruses like parvo to > 4 logs with minimum damage to valuable end-products.

Benefits

- New helical reactor type with optimum hydraulic design for uniform treatment
  - Cross-mixing with laminar flows
  - Narrow residence time distribution
  - Exact control of radiation dosage without over- or underexposure
  - No need for free-radical scavengers
  - Maintenance of an average “kill zone” even at very low transmission values
- Compact and easy to install
  - Fits into existing pipework by triclamp connectors
  - Pre-assembled systems available pre-wired and skid-mounted
- Cheap and easy to maintain
  - no CIP of reactor by “single use” concept
  - quick and simple reactor and lamp replacement
  - full automation
- Capacity from 15 up to 800 l/h, or parallel multi-reactor configuration
- Available with a variety of design features that meet the stringent criteria of the cGMP and are prepared for full acceptance by the FDA and other regulatory agencies.
UV System Features
• Hardware
  - Cabinet housing
  - High-grade finishes pipework system
  - Valves with solenoid position
  - UV lamp incl. power supply
  - Linear drive for UV lamp
  - Triple-headed diaphragm pump
  - Main power supply

• Instrumentation and control
  - PLT control with touch-sensitive panel
  - Position sensing for reactor module or dummy installation
  - Level sensor for leakage monitoring
  - Air sensor
  - Mass flow control
  - Pressure sensor
  - Temperature control
  - Conductivity sensor

• Paperless recorder for documentation
Iodoacetaldehyde technology for virus inactivation

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2-iodoacetaldehyde (2-iodoethanal, I-CH$_2$-CHO, 2-IAA) has been synthesized and investigated for its pathogen inactivating capabilities. IAA is synthesized as a dimer hydrate, which can be isolated and purified with greater than 97% purity. The compound is stable for more than 24 months when stored at 8 °C. A 50 mM solution in pure water retains its virus inactivating capacity for more than 4 weeks.

Several enveloped and non-enveloped viruses have been treated with IAA. Examples are shown in Figures 1 and 2. Especially interesting is the good effect IAA has on the hepatitis A virus (HAV), which is known to be very difficult to inactivate by pasteurization and with other inactivation technologies.

Fig 1. Inactivation of non-enveloped viruses in 50 mg IgG or HSA/ml, 50 mM NaAc, pH 5.0 with 5 mM IAA.

Fig 2. Inactivation of enveloped viruses in 50 mg IgG/ml, 50 mM NaAc, pH 5.0 with 5 mM IAA.
It is hypothesized that IAA inactivates virus by reacting with the bases A, C or G in the virus genomes. To investigate this hypothesis, free bases of 50 mM each were treated with 50 mM IAA at pH 5.5, for 16 hours at 37 °C. The reaction was followed by NMR and by mass spectrometry. In all cases investigated, shifts in NMR corresponding to the proposed etheno adduct were observed. The mass spectrometry analysis confirmed the individual etheno adducts formed (Fig 3).

To verify the action of IAA on virus genomes, one RNA viruses (Bovine diarrhea virus, (BVDV) 12.3 kb) and one DNA virus (Bovine herpes virus type 1, (BVH-1) 152 kb) were treated with IAA. Both viruses were inactivated to the limit of detection after 2 hours of treatment. The virus particles were isolated after different treatment times and the genomes subjected to different analyses. In the case of BVDV, four segments of the genome were sequenced (290, 1200, 250 and 900 base pairs long). Point mutations at A and G repeats were seen. Amplification of small PCR products was possible but unsuccessful for larger amplicons, indicating random attachment of IAA to the nucleic acid. In the case of BHV-1, two segments were analyzed (290 and 520 base pairs long). This DNA virus showed very few sequence disturbances and the two PCR amplicons were amplified with good results. In a subsequent study it was possible to utilize the fact that BVDV-RNA is able to produce infectious virus particles if introduced into susceptible cells. RNA from IAA-treated BVDV virus (2 h and 6 h) were isolated and electrophorated into fresh bovine turbinate cells, followed by cultivation. As a control, RNA from non-treated BVDV was used. On cytopathic effect, the supernatant was harvested and BT cells inoculated to confirm infectivity.

It was observed that the RNA was non-viable after treatment with IAA for 4–6 hours, and in the cell infectivity assay, complete inactivation was seen after 2 hours. One explanation for this effect could be that some proteins vital for the cell-infection are disturbed by IAA.

Both porcine parvovirus (PPV) and equine (horse) influenza virus type A-2 have a virus capsid protein (hemagglutinin, HA) that binds to sialic acid residues on the cell membrane during the early stages of a virus infection. HA titers were determined after 1, 6 and 18 hours of IAA treatment by a leukocyte agglutinating assay. The titers were unchanged compared with the control, indicating that IAA had no effect on HA that could explain the difference in BVDV-RNA infectivity and the cell infectivity assay described above.

Attempts to investigate IAA reactivity with free amino acids have shown that the only amino acids that clearly react with IAA are cystine thiol groups. No other amino acid showed reactivity when treated with an equimolar (25 mM) concentration of 25 mM IAA at pH 5 for 18 hours and analyzed by NMR.

Peptides have been treated with IAA and analyzed with both MALDI-ToF and analytical reversed phase chromatography (RPC). 0.5 mM peptide (M, 2546) was treated with 5 mM IAA, 50 mM NaAc, pH 5.0 for 48 h at 22 °C.

\[
\begin{align*}
\text{Gly-Gly-Lys-Pro-Asp-Leu-Arg-Pro-} \\
\text{Cys-Try-Pro-Pro-Cys-His-Try-Ile-} \\
\text{Pro-Arg-Pro-lys-Pro-Arg-COOH}
\end{align*}
\]

* few patches of changed cells

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**Table 1. Occurrence of viable BVDV particles after transfection of RNA from IAA treated BVDV.**

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
<th>8 h</th>
<th>virus control</th>
</tr>
</thead>
<tbody>
<tr>
<td>BVDV particles</td>
<td>viable</td>
<td>viable</td>
<td>viable*</td>
<td>non-viable</td>
<td>non-viable</td>
<td>viable</td>
</tr>
</tbody>
</table>
No changes in the mass spectrum were seen in MALDI-ToF. The RPC profile showed a slightly broader main peak and some small late eluting peaks, which corresponded to a decrease of about 0.6% in the main peak area. In another example, 50 µM Neurotensin, was treated with 2.5 mM IAA at pH 5.2 for 14 hours at 37 °C (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu, Mₐ: 1672.9) and analyzed by LC-MS/MS. The fragment pattern as well as the RPC profile were identical before and after IAA treatment, indicating that exposure to IAA does not cause any change in the peptide.

Several operational parameters have been investigated, including IAA concentration, time and temperature, influence of protein concentration, pH and ionic strength dependency.

As for all chemical reactions, the effect of IAA on virus is faster at elevated temperature and at higher concentration. Inactivation of porcine parvovirus to > 4 logs is achieved with one of the following:

- 0.5 mM IAA and incubation for 16 hours at room temperature
- 5 mM IAA and incubation for 3 hours at room temperature
- 1 mM IAA and incubation for 3 hours at 37 °C.

The reaction between IAA and virus is not protein dependent, no change in virus inactivation kinetics was seen between when only buffer or 125 mg IgG/ml was present.

The reaction is pH dependent and decreases above pH 7. At pH 8, PPV is inactivated to 60% compared with at pH 7 and below. The reaction has also been challenged with different ionic strengths; the addition of up to 750 mM NaCl has no effect on the virus inactivation reaction.

Conclusions

2-iodoacetaldehyde is an interesting new substance for virus inactivation as it inactivates both enveloped and non-enveloped viruses, and has shown to be effective against several difficult viruses including HAV. Experimental results suggest that its mechanism is the permanent modification of DNA/RNA, which disrupts virus replication. The reagent reacts with free SH groups in cysteine, but no other treatment-related effects on free amino acids or peptides has been observed. Robust virus inactivation is achieved irrespective of protein concentration or ionic strength. The effect on virus is best at pH 7 and below. Operational parameters (pH, time, temperature, buffers, IAA concentration) can be varied to suit many process conditions.
A new process for the preparation of immunoglobulin G

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Introduction

An alternative process for the preparation of immunoglobulin G (IgG) has been developed. This process incorporates the use of Gradiflow™ technology as a primary IgG capture step instead of conventional Cohn fractionation. Cohn fractionation relies on repeated ethanol precipitation under tightly controlled conditions. Subsequent filtering and resolubilization of pastes are necessary, resulting in large losses of product with yields often less than 50%.

Gradiflow is a membrane-based preparative electrophoresis system that is useful for purifying charged species from complex mixtures. Proteins in a buffer environment of a specific pH are charged according to their isoelectric points. Gradiflow utilizes this characteristic combined with size exclusion membranes to perform size- and charge-based purifications.

While Gradiflow on its own can be used to purify IgG, this study investigated the integration of Gradiflow technology into current Cohn based production schemes. Specifically, this study aimed to demonstrate the ability of the Gradiflow to purify IgG from various Cohn fraction intermediaries and the viability of scaling up the process. Simultaneous viral clearance during purification was also investigated by performing validation experiments using porcine parvovirus (PPV) as a model for human parvovirus B-19. Current viral removal techniques are often ineffective in removing parvo B-19 due to its small size and ability to withstand solvent detergent treatment.

Scale-up of the Gradiflow process was performed by increasing the separation membrane surface area to 1600 cm² (100 times that of the laboratory scale). This was largely achieved by stacking the separation unit membranes in a plate and frame format. The prototype Gradiflow instrument purified IgG from 1 liter of plasma equivalents of Cohn I filtrate and Cohn II + III paste.

Results

Laboratory scale experiments

It was demonstrated that the Gradiflow technology at laboratory scale was capable of purifying IgG from the differing feeds with yields exceeding 90%. Purity was measured to be greater than 95% by HPLC (data not shown). An SDS PAGE of these samples is shown in Figures 1 and 2. Greater than 98% of Gradiflow IgG existed as the monomer and dimeric forms, while the product also retained biological activity and normal subclass distribution. Marked reductions in levels of immunoglobulin A (IgA), immunoglobulin M (IgM) and prekallikrein activator (PKA) were achieved during the Gradiflow process.

Polishing step

A substantial reduction of contaminants was achieved during the Gradiflow process. A single, anion exchange chromatography polishing step reduced the remaining low levels of IgA, IgM, PKA and plasminogen to within the European Pharmacopoeia specifications for human normal immunoglobulin for intravenous administration.

Fig 1. Purification of IgG from Cohn I Filtrate.
SDS-PAGE 4–20% gradient gel stained with Coomassie™ blue.
[Lane 1: Wide Range Molecular Weight Marker (Sigma) indicating 205, 116, 97, 84, 66, 55, 45, 36, 29, 24, 20, 14.2, 6.5 kDa, Lane 2: Gamma P, Lane 3: Cohn I Filtrate, Lane 4: Feed after Gradiflow process, Lanes 5–10: Gradiflow IgG product after 0, 60, 120, 180, 240, 300+360 minutes respectively].
Fig 2. Purification of IgG from Cohn II + III Paste. SDS-PAGE 4–20% gradient gel stained with Coomassie blue. [Lane 1: Wide Range Molecular Weight Marker (Sigma), Lane 2: Gamma P, Lane 3: Cohn II + III paste, Lane 4: Feed after Gradiflow process, Lanes 5–10: Gradiflow IgG product after 0, 60, 120, 180, 240, 300+360 minutes respectively].

Scale-up experiments

Cohn I filtrate
The laboratory scale Gradiflow purified IgG from 10 ml Cohn I filtrate with 91 ± 2% yield (mean ± SD, n=5) in 6 hours with 96.5% purity. Using the scale-up prototype, 1 liter was processed in the same time frame and 94% yield (n=1) was achieved. SDS PAGE analysis shows that direct implementation of the bench scale protocol for scale-up operation produced a slightly different purification profile (Fig 3A). Further optimization of the scale-up process (i.e. pH 5.7 buffer instead of pH 5.4) improved the purity of the product while slightly reducing the yield (Fig 3C). This highlighted the potential of the scale-up prototype with further optimization and design modifications.

Analysis of Gradiflow products from the Cohn I filtrate scale-up experiments showed that greater than 87.7% purity was achieved, subclass distribution was typical and functional antibodies were detected. HPLC showed that the IgG polymer population of Gradiflow IgG was comparable to therapeutic IgG (Figs 3A–C). Some contaminant levels including IgA, IgM and plasminogen concentrations were higher than therapeutic IgG. However, these contaminants were successfully removed during the chromatography polishing step (Fig 3B). Without a further increase in scale-up infrastructure or optimization of input parameters, volumes of up to 10 liters of Cohn I filtrate were processed and yields of 78% were achieved in 39 hours (data not shown).

Cohn II + III paste
The laboratory bench-scale Gradiflow purified IgG from 10 ml resolubilized Cohn II + III paste containing 20 mg/ml IgG with 92 ± 3% yield (mean ± SD, n=9) in 6 hours with 98.9% purity. Using the scale-up prototype, 1 liter was processed in the same time frame and 81 ± 3% yield (mean ± SD, n=2) was achieved (data not shown).
The Gradiflow scale-up prototype was capable of purifying IgG from 2.25 liters of Cohn II + III paste (10 liters of plasma equivalent) to 86 ± 3% (mean ± SD, n=2) yield in 13 hours. Further optimization of operational parameters such as temperature, flow rates and buffer volumes has yet to be performed.

Results upon analysis of Gradiflow IgG products are similar to those achieved for Cohn I filtrate with the exception of PKA and isoagglutinin titers being higher (due to the type O source plasma used). Use of pooled plasma source material would result in much lower isoagglutinin levels. Chromatography polishing was successful in reducing the remainder of contaminants to therapeutic levels.

### Viral clearance

The Gradiflow capture step was validated for viral reduction using porcine parvovirus (PPV) as a model for human parvo B19. The levels of virus were quantified using both nested polymerase chain reaction (PCR) and infectivity assays. It was demonstrated that a PPV reduction of greater than 4.2 logs was achieved during IgG processing, which exceeds regulatory requirements for a single step. Table 1 summarizes the viral clearance results.

<table>
<thead>
<tr>
<th>Feed</th>
<th>Nested PCR (Viral log reduction)</th>
<th>TCID&lt;sub&gt;50&lt;/sub&gt; (Viral log reduction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohn II + III paste</td>
<td>4.2</td>
<td>&gt; 4.9</td>
</tr>
<tr>
<td>Cohn I filtrate</td>
<td>6.2</td>
<td>&gt; 4.9</td>
</tr>
</tbody>
</table>

### Conclusion

This study demonstrated that a single step Gradiflow purification process combined with a simple chromatography polishing step was capable of producing a therapeutic grade IVIG product consistent with European Pharmacopoeia guidelines. The viability of scale-up was proven at approximately 100 and 1000 times that of the laboratory scale instrument with volumes equivalent to up to 10 liters of plasma processed. With the system and purification protocol not yet fully optimized at large-scale, IgG yields ranged from 75 to 94% with purities at 88 to 99%. Further optimization and process development are expected to result in a system capable of incorporation into a large-scale IVIG manufacturing scheme.

With the evident advantages of high yield and purity, combined with the ability to reduce if not completely remove any pathogenic burdens, this technology is evolving as a genuine contender to modernize the plasma fractionation industry.
IgG purification using IEX filter technology

Introduction

Ion exchange (IEX) filter technology has found a role in the manufacture of recombinant products for the removal of residual cell debris and DNA. Applying the technology to plasma protein purification has a number of potential benefits. The accessibility of the functional groups allows for rapid protein binding even at high flow rates. While disposability removes the need for cleaning validation. In this study the ability of the Mustang™ Q filter (Pall Corporation) to purify IgG from an immunoglobulin enriched feedstock was investigated.

Purification of IgG using Mustang Q

The chromatographic cycle was conducted in an analogous manner to a resin-based chromatography step with the filter initially equilibrated prior to protein loading, washing, elution and regeneration. Under the conditions chosen, IgG passed through the filter while contaminant species such as IgA and IgM bound to the filter (Fig 1). The binding capacity of the Mustang Q filters for the crude IgG was initially determined using 0.35 ml ‘coin’ disc filters. However, the filters readily blocked and even with 1 M NaOH washes, repeated cycles resulted in the development of increased back-pressure. Therefore cartridge filters (Novasip CL5 – 10 ml membrane volume) were used for the dynamic binding studies. Feedstock was loaded onto the CL5 filter and fractions tested for the appearance of IgA and IgM. IgA breakthrough occurred at a loading of 150 mg of protein per ml of filter membrane volume. The results indicated that IgA levels in the purified IgG were reduced by at least 10 fold, with IgM levels reduced to below detection limits. A washing phase was required to maximize IgG recovery. This step required multiple filter volumes and was associated with the elution of IgA that resulted in higher IgA levels in the final product (Fig 2). Recovery of IgG was determined to be in the range of 80 to 90%.

Filter design – dispersion effects

Novasip CL5 filters are designed with relatively large inlet and outlet dead volumes (90 ml) compared with the membrane volume (10 ml). This facet of the filters was thought to be a major contributor to the long equilibration and washing phases in the chromatographic cycle. Mathematical modeling using the system dispersion model of Sarfert and Etzel was undertaken to determine the contribution of these dead volumes on the chromatographic separation (1). The model assumes that dispersion consists of an ideal plug-flow reactor and a continuously-stirred tank reactor in
series (Equation 1). Initially the model was fitted to conductivity profiles obtained for the 1 M NaCl step used in the Mustang Q chromatographic cycle. Using parameters \( V_{PFR} \) & \( V_{CSTR} \) derived from the 1 M NaCl conductivity curve, the model was then used to predict the IgG elution profiles (Fig 3). The modeling provided a good approximation of the experimental IgG drop-through curves and therefore provided evidence that dispersion effects account for the peak broadening observed during the washing phase.

\[
C_{out}(t) = \begin{cases} 
0 & \text{for } t < \frac{V}{f} \\
\int_{0}^{t} \frac{f}{V_{cor}} \exp\left(-\frac{ft}{V_{cor}}\right) C_{in}(t) \exp\left(\frac{ft}{V_{cor}}\right) dt & \text{for } t \geq \frac{V}{f}
\end{cases}
\]

Equation 1. Mathematical system dispersion model of Sarfert and Etzel. PFR – ideal plug flow; CSTR – continuously stirred tank reactor; \( C_{out} \) - outlet concentration; \( C_{in} \) - inlet concentration; \( V \) - volume; \( f \) – flow rate; and \( t \) – time.

In a production system it is likely that the dead volume to membrane volume ratio would be reduced. Under these circumstances the dispersion effects would be diminished, resulting in reduced buffer consumption, shorter equilibration and wash times, and a higher final IgG concentration in the wash through fraction. The result highlights the difficulties that can be experienced during scale-down evaluation of process steps.

**Effect of conductivity on retention of bound proteins during washing**

The increased levels of contaminant IgA in the wash fraction provide some evidence to indicate that bound species may have been leaking from the filter. This observation may be explained by the fact that proteins do not irreversibly bind to the ligands on ion exchangers, rather they remain in a dynamic equilibrium continually binding and unbinding to the ligands. Switching to the washing solution alters the equilibrium, which results in higher contaminant concentrations eluting from the filter. In contrast, proteins in resin-based chromatography remain on the column during washing phases due to the slower intra-particle mass transfer rates and reduced dispersion effects. The Mustang Q contains only 16 layers of membrane and consequently the path length available to proteins is reduced. Thus during the washing phase protein will move from binding site to binding site and eventually from the filter.

In an attempt to reduce the IgA levels eluting from the filter during the washing phase, a study was conducted using different washing solutions (water, 10 mM and 50 mM sodium acetate). The results indicated that as the conductivity of the washing solution increased, the levels of IgA in the drop-through increased. It is therefore recommended that the lowest practical concentration of buffering agent be used to wash product from the filter.

**Performance of Mustang Q over multiple cycles**

The effect of repeated cycles on Mustang Q performance was conducted over 8 cycles. The method used a 1 M NaCl strip to remove residual protein from the filter between cycles. The drop-through displayed a consistent profile for all cycles but a broadening of the NaCl peak was observed. Although the IgG profiles were very similar a substantial increase in IgA concentration from cycles 1 to 8. Therefore it is recommended that a 1 M NaOH step be incorporated after the 1 M NaCl strip to maximize protein removal from the filter between cycles.

Using this approach the ability of the Mustang Q filter to process multiple cycles was assessed over 30 cycles. The pH, conductivity and UV from the cycles remained consistent over the 30 cycles. Analysis of IgA levels in the 1–15 cycle pool compared to the 16–30 cycle pool indicated that the capacity to remove IgA diminished with increasing cycle number. Similarly, the IgM levels also increased in the 16–30 cycle pool. It is likely that the reduced performance over the cycles 16–30 is related to filter blockage reducing the overall number of Q-ligand binding sites available to the IgA and IgM.
Conclusions
The studies indicate the IEX filter technology is a potential alternative to resin-based chromatographic procedures to remove trace contaminant species while achieving a satisfactory protein recovery. The use of these filters requires multiple cycles. These studies demonstrated that this is possible. A limitation identified with multiple cycles is that the filter will become progressively blocked resulting in a reduction in the total binding capacity of the filter. Therefore it is necessary to load the filter at levels that will not exceed the binding capacity through the entire cycle set. Further, pre-filtration of the feedstock is likely to be a critical parameter for minimizing the occurrence of this blockage.

The major difference between resin-based chromatography and the filter technology was the long equilibration and wash phases and the increased contaminant levels in the wash. These issues are related to filter design and choice of washing solution. Lower conductivity wash solutions promote retention of bound contaminating proteins on the filter.

The development of charged filter technology as an alternative to resin-based chromatographic procedures will be dictated by the purification requirements of the process in question and will require specific optimization work.

Reference
The regulatory frame to assess the viral safety of medicinal products was established in Europe more than 10 years ago. However, cases of virus transmission from plasma derivatives in the early 90s clearly demonstrated that this frame was insufficient. More extended Guidance documents were therefore developed and brought into force in 1995. The regulatory framework was also extended to other categories of products, cell-derived products, and products made from animal sources like immune sera and immunoglobulins. The industry also developed new models for the production of therapeutic proteins: transgenic animals and transgenic plants. These and other products can be summarized under the term Biologicals.

Biologics carry the risk of virus contamination of the source material. The viral safety of these products depends on the relationship between the possible level of contamination of the source material and the capacity of the process to remove and inactivate viruses. Several European or International Guidelines are in place which give recommendations for source material testing, as well as for investigating and demonstrating the capacity of manufacturing process to remove and inactivate virus (1,2,3). The level of safety of the source material varies; risks may be highest in the case of plasma derivatives and lowest in the case of cell-derived products where no animal derived materials are used for fermentation. This may be illustrated by the following:

It is well known that there is a risk that human plasma is contaminated with viruses: of most concern are the human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), while hepatitis A virus (HAV) and parvovirus B19 contamination are of concern for some products (4). Donor selection and testing of each donation are therefore required (2,5). In order to improve the data demonstrating the safety of the source material, the regulatory tool Plasma Master File (PMF) was established in Europe (2). Two important parts of the PMF are: reporting the origin of plasma (establishments, testing sites, traceability from plasma to finished product and vice versa) and presenting data on plasma quality and safety (testing of donation and pools, conditions of storage and transport, inventory hold). A proposal for the scientific data requirements for a PMF has been recently published (7).

To assure the safety of human plasma, it is necessary to analyze continuously the epidemiological situation. The West Nile virus (WNV) epidemic in the USA and the occurrence of severe acute respiratory syndrome (SARS) in several places in the world are recent examples demonstrating that additional requirements may be needed. From July 2003, the FDA recommends testing of donations used for preparation of cellular components (erythrocytes, platelets, plasma for transfusion) on the presence of WNV infection (8). On the basis of existing validation data for enveloped viruses, it can be anticipated for plasma-derived medicinal products that the inactivation/removal steps presently incorporated in manufacturing processes will also be effective for WNV (9). The occurrence of SARS initiated the introduction of specific donor exclusion criteria in some countries (10,11). Furthermore, the theoretical risk of contamination of human plasma with new variant Creutzfeldt-Jacob Disease (vCJD) is subject to permanent review by regulatory authorities (12).

In the case of cell-derived medicinal products, viruses may be immanent in the cell line (an example is the presence of retroviruses or retrovirus-like particles in rodent cells, such as CHO or BHK cells), may be introduced by medium components (most important is fetal bovine serum (FBS)), or may be introduced by personnel or materials used in the purification process. Even porcine trypsin, which is normally only tested on absence of porcine parvovirus, carries the risk of contamination. Bovine serum or FBS was the source of several cases of
virus transmission through veterinary vaccines, and fermentor contamination was observed in other cases. Several viruses are frequent serum contaminants (Table 1). Although the testing of each batch of serum is required (13,14), contamination cannot be completely avoided, especially where bovine viral diarrhea virus (BVDV) and bovine polyoma virus (BPyV) are concerned. To reduce the risk of contamination, the use of inactivated serum is required (13) or highly recommended (14). Inactivation by gamma irradiation is recommended.

Table 1.

<table>
<thead>
<tr>
<th>Virus/strain</th>
<th>Family</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine viral diarrhea virus</td>
<td>Flaviviridae, enveloped, RNA</td>
<td></td>
</tr>
<tr>
<td>Parainfluenza virus, Type 3</td>
<td>Parainfluenzaviridae, enveloped, RNA</td>
<td></td>
</tr>
<tr>
<td>Infectious bovine rhinotracheitis virus</td>
<td>Herpesviridae, enveloped, DNA</td>
<td></td>
</tr>
<tr>
<td>Cache valley virus</td>
<td>Bunyaviridae, enveloped, RNA</td>
<td></td>
</tr>
<tr>
<td>Bluetongue virus</td>
<td>Reoviridae, non-enveloped, RNA</td>
<td></td>
</tr>
<tr>
<td>Epizootic haemorrhagic disease virus</td>
<td>Reoviridae, non-enveloped, RNA</td>
<td></td>
</tr>
<tr>
<td>Bovine polyoma virus</td>
<td>Papovaviridae, non-enveloped, DNA</td>
<td></td>
</tr>
</tbody>
</table>

To characterize the safety of the cell line used for production, cells of the Master Cell Bank and cells at the end of production must be monitored for retroviruses, or retrovirus-like particles, and adventitious viruses using in vitro and in vivo assays (3).

As the cell culture system is beneficial for virus replication, the manufacturing process for cell-derived products must therefore have a sufficient high capacity for virus inactivation (3). The lower the safety level of the cell culture system, the higher the capacity of the manufacturing process must be.

If animals are used for production of medicinal products, the animals need to be monitored for viral infection. This may be done by clinical observation, by testing of sentinel animals, or by routine control of breeding and/or production animals. The requirements for testing are higher if the animals are not kept in closed herds isolated from contact with wild animals. This may be the case if larger animals like horses or goats are used for production or for development of transgenic animals. Animal sera and immunoglobulins are presently produced in horses, goats or sheep, and rabbits. "The Note for Guidance on production quality control of animal immunoglobulins and immunsera for human use" (15) provides species-specific lists of viruses which need to be considered if these species are used. There are no requirements in place at present for testing transgenic animals (16), but the lists given in the guideline mentioned above may be applied. If other species are used, manufacturers should perform a risk assessment.

If virus testing is applied to demonstrate the safety of the source material, the intermediate, or the final product, it should be considered that testing has limitations even from a statistical standpoint and cannot assure the absence of infectious virus. If we assume that the test system is highly sensitive and can detect each pathogenic particle (e.g. PCR), the sample size is decisive for the detection limit. The Poisson distribution should be applied and it shows that in order to detect a contamination level of 10 virus particles per ml with a probability of 95%, 0.3 ml must be tested. Even if 300 ml of the product or intermediate have been tested with a negative result, we can only assume with 95% probability that the product does not contain more than 1 infectious particle per 100 ml. This would mean that 10 vials in a batch of 1000 vials can be infectious (Table 2).

Table 2. Detection of low virus load by testing.

<table>
<thead>
<tr>
<th>Virus/ml</th>
<th>Volume tested [ml]</th>
<th>Poisson distribution: $\ln p(-) = c \times v$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.3</td>
<td>p = 0.05</td>
</tr>
<tr>
<td>1.0</td>
<td>3.0</td>
<td>p = 0.01</td>
</tr>
<tr>
<td>0.1</td>
<td>30.0</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>0.01</td>
<td>300.0</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>0.001</td>
<td>3000.0</td>
<td>p = 0.001</td>
</tr>
</tbody>
</table>

This example illustrates the fact that testing cannot assure the absence of infectious particles in the material tested and the capacity of the manufacturing process to remove or inactivate viruses is critical for the viral safety of the product. Requirements have been developed for cell-derived products as well as for products derived from human or animal material (1,3).
In designing virus experiments the following questions should be answered:

**Analysis of the production process:**
- What is the safety profile of the source material?
- Which steps may have a capacity for virus removal or inactivation?
- Which process parameters may influence virus removal/inactivation?

**Defining an appropriate study design:**
- How to transform the indicated steps into the laboratory scale?
- How to demonstrate comparability between the manufacturing and the laboratory processes?
- Which viruses are appropriate for reflecting product safety and should be used for performing viral clearance studies?
- Which virus assays should be used?
- Which spike material is appropriate?
- Which process parameters have to be investigated to demonstrate robustness of virus removal/inactivation?

Virus experiments are performed at laboratory-scale. The transfer of the manufacturing conditions to the laboratory experiments must be demonstrated by comparing process parameters as well as analytical data of intermediates. The reliability of the down-scaling is crucial and is as important as the virus experiments themselves for demonstrating that the virus results can be accepted as representative for the manufacturing process. Deviations from the manufacturing conditions which cannot be avoided must be analyzed with respect to their influence on virus removal or inactivation.

The following methods are currently used for the manufacture of medicinal products (Table 3). Fractionation (precipitation, chromatography, depth filtration) may have some capacity for virus removal. The capacity may be product and virus related. An effective method for virus removal is ‘nanofiltration’. 'Nanofiltration' is the use of filters which are optimized for virus retention. This method has the highest capacity for virus removal if filters of the smallest pore size can be used. Effective separation of viruses from the product is possible if the product has a smaller molecular size than the virus in question. In such cases, virus will be retained while the product passes through the membrane.

Heat treatments and solvent/detergent methods are used mostly for inactivation of plasma-derived medicinal products. Low pH treatment (<pH 4) is effective for inactivation of retroviruses and is often used in the manufacture of cell-derived medicinal products.

Photochemical and photodynamic methods are under development and may be used in the future for inactivation of cellular components; application of gamma irradiation (≥ 50 kGy) for virus inactivation of medicinal products may also provide an effective tool for virus inactivation of plasma derivatives or recombinant products. First results demonstrate that product integrity can be maintained during irradiation at high dose where pathogens are inactivated.

<table>
<thead>
<tr>
<th>Table 3. Methods for manufacture of medicinal products.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fractionation/virus removal:</strong></td>
</tr>
<tr>
<td>• Precipitation</td>
</tr>
<tr>
<td>– Ethanol, PEG</td>
</tr>
<tr>
<td>• Chromatography</td>
</tr>
<tr>
<td>– Ion exchange</td>
</tr>
<tr>
<td>– Affinity</td>
</tr>
<tr>
<td>– Hydrophobic interaction</td>
</tr>
<tr>
<td>• Filtration</td>
</tr>
<tr>
<td>– Depth filtration</td>
</tr>
<tr>
<td>– Nanofiltration</td>
</tr>
</tbody>
</table>
The purpose of virus experiments is to demonstrate the real capacity of the manufacturing process to remove/inactivate a broad range of viruses and provide data demonstrating the robustness of the individual steps for virus removal/inactivation. The inactivation/removal mechanism needs to be evaluated. Individual reduction values are summed to an overall reduction value which should then give an indication of the overall capacity of the process. To clarify whether reduction values are additive or not, it may be helpful in some cases to perform additional virus removal tests in combined experiments; virus is first spiked separately into each step, then the removal capacity of each step is confirmed by spiking once into the intermediate and analyzing virus partitioning after each step. At the end of the evaluation questions need to be answered: is the product safety acceptable and sufficiently documented?

For newly developed treatment strategies that need specific regulations there are guidance documents to assure safety and efficacy of treatments and products. Gene therapy and somatic cell therapy are examples. Transgenic plants and transgenic animals are now established as tools for production of recombinant products and guidance documents have been developed for these product categories (19,20,21,16).

The risk of transmissible spongiform encephalopathies (TSE) transmission by human plasma-derived or urine-derived medicinal products has been under review by regulatory agencies for several years (22). The CPMP issued an updated position statement in February 2003 (12) which summarized the regulatory consequences from current knowledge; this is briefly outlined below:

- It is finally not known whether infectivity is in blood or not.
- Available data indicate that manufacturing processes would reduce infectivity, if it were present. Product related investigational studies are in progress.
- UK decided not to fractionate UK plasma. It is prudent to exclude donors with a long residence time in UK: cumulative 1 year between 1980 and 1996.
- PrPsc in urine has been reported; no infectivity found so far, further investigation is needed; donor exclusion is not recommended.

In summary, the level of assurance for the viral safety of plasma and biotech products is at present high. Regulatory agencies provide Guidance documents that afford a basis for establishing and characterizing the safety of the products in place.

New treatment strategies and newly developed products categories are supported by some guidance documents, but this area needs further consideration and support by the agencies.

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Abbreviations:

EMEA The European Agency for the Evaluation of Medicinal Products

CPMP Committee for Proprietary Medicinal Products
Immunomodulatory effects of intravenous immunoglobulin in autoimmune and inflammatory diseases

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Intravenous immunoglobulin (IVIG) has increasingly been used for the treatment of autoimmune and systemic inflammatory diseases. The number of diseases in which the effect of IVIG therapy has been demonstrated by large-scale, controlled clinical trials remains limited. Available clinical and experimental evidence suggests, however, that a wide spectrum of immune-mediated conditions could benefit from IVIG, including acute and chronic/relapsing diseases, autoimmune diseases mediated by pathogenic autoantibodies or by autoaggressive T cells and inflammatory disorders associated with, for example, an imbalance in cytokine networks.

The list of disorders reportedly responding to IVIG has been growing in recent years. It now includes a wide spectrum of diseases mediated by pathogenic autoantibodies or believed to be primarily dependent on autoaggressive T cells. In only few of the diseases has the beneficial effect of IVIG been established in prospective randomized clinical trials (Table 1). In many other conditions, the effects of IVIG have been documented in uncontrolled studies, including anti-factor VIII autoimmune disease, warm autoimmune hemolytic anemia, autoimmune erythoblastopenia, autoimmune neutropenia, polymyositis, the antiphospholipid syndrome, recurrent spontaneous abortions, SLE, thyroid ophtalmopathy, and Crohn's disease. Our current knowledge is still insufficient as to how IVIG should be optimally administered for immunomodulation, e.g. aiming at keeping high plasma concentrations of immunoglobulin for prolonged periods of time or, alternatively, spiking the immune system with intermittent high doses of immunoglobulin.

Commercially available IVIG consists of intact IgG molecules with a distribution of IgG subclasses that corresponds to that of normal human serum. Most preparations contain traces of IgA and carry the risk of sensitization to IgA in the long-term treatment of IgA-deficient individuals. IVIG has also been shown to contain trace amounts of soluble CD4, CD8 and HLA molecules and of certain cytokines such as TGFβ. The half-life of infused IVIG in immunocompetent individuals is three weeks. The preparations contain intact Fc moieties which allow IVIG to interact with and signal through Fc receptors on Fc receptor-expressing cells, including phagocytes and B lymphocytes, and with a number of Fc-binding plasma proteins, e.g. components of the complement system.

Since it was first used in the treatment of idiopathic thrombocytopenic purpura, considerable progress has been made in understanding the mechanisms by which IVIG exerts immunomodulatory functions. The mode of action of IVIG is complex, involving modulation of expression and function of Fc receptors, interference with activation of complement and the cytokine network, provision of anti-idiotypic antibodies, inhibition of maturation and function of DC, regulation of cell growth, and

Table 1. Autoimmune and inflammatory diseases in which the beneficial effect of IVIG has been established in controlled clinical trials.

<table>
<thead>
<tr>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune thrombocytopenic purpura</td>
</tr>
<tr>
<td>Guillain Barré syndrome</td>
</tr>
<tr>
<td>Chronic inflammatory demyelinating polyneuropathy</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
</tr>
<tr>
<td>Multifocal motor neuropathy</td>
</tr>
<tr>
<td>Steroid-resistant dermatomyositis</td>
</tr>
<tr>
<td>Kawasaki disease</td>
</tr>
<tr>
<td>Graft versus host reaction</td>
</tr>
<tr>
<td>ANCA-positive vasculitis*</td>
</tr>
<tr>
<td>Autoimmune uveitis*</td>
</tr>
<tr>
<td>Multiple sclerosis*</td>
</tr>
</tbody>
</table>

* First results have been published; major controlled trials underway.
effects on the activation differentiation and effector functions of T and B cells (Table 2). The ability of IVIG to interact through V regions with complementary V regions of antibodies and antigen receptors as well as with relevant soluble and surface molecules provides the basis for inducing the selection of immune repertoires (see Table 3).

Modulation by IVIG of the production of cytokines and cytokine antagonists is a major mechanism by which immunoglobulin exerts its anti-inflammatory effects in vivo. In in vitro experiments, IVIG was shown to selectively trigger the production of interleukin-1 receptor antagonist (IL-1ra), the natural antagonist of interleukin-1, in cultures of purified monocytes, without concomitant effect on the production of the proinflammatory cytokines interleukin-1α, interleukin-1β, interleukin-6 and tumor necrosis factor-α (TNF-α). Interleukin-1ra had originally been identified in supernatants of cultures of human monocytes on immobilized human IgG. Decreased levels of interleukin-1 after infusion of IVIG in patients with Kawasaki disease, and increased plasma levels of IL-1ra with a 1000-fold molar excess of IL-1ra over interleukin-1β have been reported following administration of IVIG. Circulating levels of interleukin-1β decrease after patients with the Guillain-Barré syndrome receive IVIG treatment. The anti-inflammatory effects of IVIG relating to modulation of cytokine production are not restricted to monocytic cytokines, but are also largely dependent on the ability of IVIG to modulate T helper 1 and T helper 2 cytokine production.

Analysis of the immunoregulatory effects of IVIG sheds light on the functions of natural antibodies that constitute a significant component of the immune system. IVIG as it is currently used takes advantage of the array of variable regions that characterizes normal polyspecific immunoglobulin and that is enhanced in IVIG preparations by the large number of donors contributing to the pool. It may be speculated that by increasing the content of specific natural autoantibodies in immunoglobulin preparations, novel means of immunomodulation with intact polyclonal human IgG will become available for the treatment of autoimmune diseases.

### Table 2. Immunoregulatory effects of IVIG.

| Fc receptors | Blockade of Fc receptors on macrophages and effector cells |
|             | Antibody-dependent cellular cytotoxicity |
|             | Induction of inhibitory FcγRIIB receptors |
| Inflammation | Attenuation of complement-mediated damage |
|             | Decrease in immune complex-mediated inflammation |
|             | Induction of anti-inflammatory cytokines |
|             | Inhibition of activation of endothelial cells |
|             | Neutralization of microbial toxins |
|             | Reduction in steroid requirements |
| B cells and antibodies | Control of emergent bone marrow B cell repertoires |
| Negative signaling through Fcγ receptor | Selective down-regulation/up-regulation of antibody production |
|             | Neutralization of circulating autoantibodies by anti-idiotypes |
| T cells | Regulation of T helper cell cytokine production |
|             | Neutralization of T cell superantigens |
| Cell growth | Inhibition of lymphocyte proliferation |
|             | Regulation of apoptosis |

### Table 3. Antibody reactivities present in IVIG against molecules and cells of the immune system.

| Variable regions of immunoglobulins | Idiotypic determinants of immunoglobulins |
|                                     | Self-binding locus of S107/T15+ immunoglobulin |
|                                     | Framework and variable determinants of the β chain of the αβ T cell receptor |
| Cytokines and cytokine receptors | CD4 |
| CD5 | MHC class I-derived peptides |
| RGD-expressing adhesive proteins | Fas |
| CCR5 |
Recombinant adenoviruses used in gene therapy cause massive complement activation via antibody induced in previous infections

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In the last two decades, great expectations were raised for gene therapy approaches to a number of diseases. The progress made in several experimental models seemed to suggest great promises for numerous patients. However, over the last few years it is now known that some of these promises were premature and not all expectations have been fulfilled. The most important vector system currently used in gene therapy is recombinant adenoviruses. Adenovirus is a common infectious agent causing frequent infections of the upper respiratory tract. Antibodies to adenovirus are found in up to two thirds of the population. Thus, if adenovirus is used for gene therapy, there is a high probability that immune complexes will form because of the pre-existing antibodies to adenoviruses. Immune complexes in turn are able to cause complement activation.

A number of experimental approaches in gene therapy have focused on adenoviruses. This is because they are highly efficient as a vehicle for gene therapy, and a broad spectrum of therapeutic strategies in gene therapy is dependent on them. The potential application of adenovirus ranges from gene therapy for compensation of genetic deficiencies to cancer therapy. A major problem in this field was revealed through a study in Philadelphia. The case of Jesse Gelsinger in this study, a couple of years ago, attracted the attention of the media. This young patient had an enzyme deficiency affecting liver function. However, this dysfunction was not of vital importance and he might have survived with appropriate diet. The patient was included in a gene therapy program and received about $10^{11}$ Ad5 (Adenovirus serotype 5) particles/kg via hepatic artery infusion. He died 3 days later due to multiple organ failure; he was 18 years old. Other patients survived. This unfortunate event caused serious doubts about the feasibility of gene therapy.

One of the humoral effector systems involved in the side effects observed in this gene therapy trial might be the complement system. The capacity of adenovirus to activate this system of plasma proteins might have been underestimated, as well as the extent of complement activation in the patient’s plasma. The marked complement activation leads to the generation of biologically highly active complement fragments, which might have contributed to the observed organ damage. These complement-split products are potent inflammatory mediators, affecting a number of cell types. Illness caused by complement activation is observed in a number of clinical conditions, leading eventually to adult respiratory distress syndrome and followed subsequently by multiple organ failure. This includes sepsis, aspiration pneumonia, polytrauma, pancreatitis, severe burn, extracorporeal circulation or haemodialysis.

To analyze the activation of complement by adenovirus, the generation of the anaphylatoxin C3a was used as parameter for complement activation. The complement component C3 is cleaved into C3a and C3b by proteolytic C3-cleavage in the course of complement activation. The generated C3a is subsequently transformed by cleavage of the C-terminal arginine to the C3a-desArg variant. We previously described a novel ELISA system using a monoclonal antibody selectively recognizing a C3a-desArg neoepitop that allows the selective measurement of C3a-desArg in the presence of native C3.

The level of C3a-desArg generated in isolated citrate plasma of healthy individuals was determined after challenge tests with recombinant or wild-type adenoviruses, in amounts corresponding to viral blood levels as they are expected in patients during adenoviral gene therapy. Substantial and dose-dependent generation of C3a-desArg was observed in all plasma samples that contained
antibodies to adenovirus. Complement activation was blocked by the addition of EGTA, known to inhibit complement activation via the classical pathway, i.e. activated by immune complexes. In addition, depletion of IgG from the plasma similarly blocked complement activation. Reconstitution with purified IgG from the corresponding plasma compensated for this effect, and the ability to cause complement activation by adenovirus serotype 5 was restored. Complement activation was observed after incubation with different adenovirus serotypes (Ad1, Ad3, Ad5, and Ad9, but not Ad4). The virus level reached in clinical trials in the past was about $7.5 \times 10^9$ particles/ml as the highest plasma level. A corresponding virus concentration in vitro caused the generation of about 3000 ng/ml C3a-des Arg; the baseline levels of C3a-desArg in the absence of deliberate complement activation was in the range of less than 140 ng/ml. The nature of the antibodies to adenovirus was analysed. Not only antibodies with neutralizing capacity (anti-Ad5), but also non-neutralizing antibodies to adenovirus were able to cause efficient complement activation.

These findings suggest that complement activation can be ignored in local, low-dose applications of recombinant adenoviruses. However, careful attention is warranted after systemic application of large viral quantities that may be required to achieve a sufficient gene transfer in the corresponding patient, according to the corresponding clinical protocols. Measuring the capacity of the genetically modified adenovirus to cause complement activation in a kind of bedside test might therefore be helpful and informative. This precautionary measure might prevent the previously observed multiple organ failure and even death in gene therapy patients.

Reference

ZLB Bioplasma has developed a reconstituted HDL (rHDL). It was prepared from human apolipoprotein A-I and soybean-derived phosphatidylcholine (PC) via cholate dialysis. Reconstituted HDL contains apoA-I and PC in a molar ratio of 1:150. The apoA-I was purified from waste fractions from the production of albumin and immunoglobulin from human plasma and included two validated virus removal/inactivation steps.

Reconstituted HDL was lyophilized in the presence of sucrose and was shown to be stable for several years. It was manufactured under Good Manufacturing Practice (GMP) conditions; the biochemical and biophysical properties of the product were well characterized. Reconstituted HDL fulfils the requirements for an intravenously compatible biological product. The manufacturing process is shown in Figure 1. The particles formed were disk shaped and resembled nascent HDL (Fig 2).

Reconstituted HDL exhibited a range of activities which suggested areas of clinical application. It promoted the mobilization of cholesterol and stimulated the cholesterol efflux from human endothelial cells, and from a monocyte-macrophage cell line. A stimulation of the cholesterol transport was also observed in animals and in clinical Phase I studies.

A range of anti-inflammatory activities was exhibited by rHDL with down-regulation of CD11b/CD18 cells, leading to the inhibition of the PMN binding to fibrinogen, and inhibition of TNF production following LPS stimulation. The anti-LPS activity of rHDL was exemplified by its capability to bind LPS in the presence of plasma and the removal of LPS from host cells. Reconstituted HDL also enhances anticoagulant activities of protein S and activated protein C, and reduced platelet activity by binding GP IIb-IIIa.

Other mechanisms of action of rHDL included enhancing bioavailability of NO, anti-oxidative effects, inhibition of adhesion molecule expression

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**Reconstituted HDL, a novel drug for acute cardiovascular, cerebrovascular, and inflammatory diseases**

Peter Späth, Alphonse Hubsch, Stefanie Koch, Bernard Spörri, Joseph Bertolini, Peter Lerch.

ZLB Bioplasma AG, Berne, Switzerland and CSL Bioplasma, Broadmeadows, Victoria, Australia

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**Fig 1.** The manufacturing process for rHDL.

**Fig 2.** Illustration of rHDL disc and electronmicrograph of discs.
and modulation of endothelial cell function, and prevention of apoptosis. These mechanisms suggest that rHDL could have many clinical applications, in particular in the treatment of atherosclerotic conditions and for neuroprotection in ischaemic/reperfusion events as may occur in stroke.

The clinical potential of rHDL is supported by a number of Phase I and II clinical trials. In 13 separate clinical studies more than 150 subjects/patients were exposed to rHDL, including healthy male volunteers, hypercholesterolemic men, critically ill children suffering from meningococcal septic shock, as well as men and women hospitalized for an acute coronary syndrome. These studies showed that administration of rHDL was safe and well tolerated, and confirmed and extended the preclinical data. Thus rHDL was shown to reduce the clinical sequelae, proinflammatory cytokine response, and procoagulant activities induced by LPS administration in healthy volunteers. A proof of concept study in hypercholesterolemic men demonstrated that rHDL at 80 mg/kg/4h acutely restored endothelial function, as determined by the increased blood flow in forearm plethysmography studies.

The most attractive indication for rHDL is in the treatment of acute ischemic stroke. Stroke ranks as the third leading cause of death and remains the leading cause of serious, long-term disability in the western world. Each year in the USA, 600,000 people suffer a new or recurrent stroke. A neuroprotective drug would be expected to protect brain tissue from the detrimental effects of acute ischemia and reperfusion. The narrowing or blockage of brain arteries as the result of sudden vascular events, such as local rupture of an atherosclerotic plaque, can produce an acute ischemic stroke. The resulting inadequate blood flow deprives the brain of oxygen and glucose. In the ischemic core, the area totally deprived of blood flow, the neurons die within minutes. In the surrounding penumbra, blood flow is reduced to a level that interrupts neuronal function, yet permits the cells to survive. Cells in the penumbra are potentially salvageable by reperfusion within 48–72 hours. There are currently no recognized effective neuroprotective agents. The rationale for developing rHDL as a neuroprotective drug is based on its multiple pharmacological profile:

- rHDL increases the bioavailability of endothelial nitric oxide (NO), which in turn could increase cerebral collateral circulation
- rHDL inhibits the expression of adhesion molecules and exhibits antioxidative properties, which may reduce the ischemic inflammatory cascade
- rHDL inhibits platelet aggregation, which may attenuate the formation of new clots in affected blood vessels

One can envisage rHDL used alone or in combination with thrombolytic agents. The later approach could extend the time window for thrombolysis treatment, thereby offering the prospect for this treatment to be more commonly applied.

In rat models it was shown that rHDL reduces infarct size and improved functional outcome when administered within 6 hours after the onset of ischemia.

Figure 3 shows that the administration of rHDL significantly reduced stroke induced damage in the rat brain.

The establishment of rHDL as a potential treatment of stroke now requires clinical assessment in human subjects.

Fig 3. Attenuation of middle cerebral arterial occlusion induced infarct with administration of rHDL.
Alpha Chromatography (Alpha-C) is an improved process for the purification of Alpha-1 Protease Inhibitor (A1-PI) from human plasma. Bayer began producing its first version of A1-PI, Prolastin™, more than 15 years ago. Prolastin has proven to be safe and effective for treating thousands of A1-PI deficient patients. When designing the improved process for the purification of A1-PI, the requirements were higher purity, increased assurance of viral safety and higher yield (Fig 1).

Step has been shown to effectively remove prion protein which is believed to cause transmissible spongiform encephalopathies (new variant CJD). Inactivating enveloped viruses with hydrophobic molecules such as Tri-N-butyl phosphate (TNBP) or octanoic acid (caprylate) results in significant loss of A1-PI activity, as measured by elastase inhibition. Since A1-PI has no disulfide bridges and relies on internal hydrophobic interactions to maintain tertiary structure, we postulate that small hydrophobic virus inactivation agents disrupt the internal hydrophobic interactions of A1-PI.

Tween 20, a nonionic detergent, was tested as an agent to inactivate lipid enveloped viruses. Tween 20 proved to be effective at rapidly inactivating enveloped viruses. Inactivation to detection limits required approximately 1 hour or less at 25 °C for all model viruses tested. In addition A1-PI was stable in Tween 20. We postulate that the larger size of Tween 20 compared to TNBP or caprylate prevents Tween 20 from infiltrating the interior (hydrophobic) section of the A1-PI molecule. Tween 20 treatment was substituted in the Alpha-C process for the pasteurization step of the Prolastin process.

The PEG filtrate was incubated with Tween 20, the material was then applied to an anion exchange column to remove transferrin and albumin as well as residual ceruloplasmin and AT-III. Because of improvements in chromatography media the PEG precipitation step is not needed and the PEG filtrate can be applied directly to an anion exchange column. The anion eluate was ultrafiltered and passed through a cation column. The cation column improves on the Prolastin process by removing the remaining albumin and IgA. As a final virus safety step, the cation flow through was then passed through a nanofilter as the third virus reduction step. The nanofilter removes virus as small as
porcine parvo (approximately 20 nanometers). The flow diagram for the process is shown in Figure 2.

The Alpha Chromatography process has three main purification steps: PEG precipitation, anion chromatography, and cation chromatography. The process also includes three significant pathogen removal steps: PEG Precipitation, Tween™ 20 Treatment, and Nanofiltration. By comparison the Prolastin process has two primary purification steps, the first PEG filtration and anion chromatography. In the Prolastin process virus clearance is attained in the first PEG filtration and by pasteurization. The yield increase seen in the Alpha-C process is attributable to replacing the pasteurization step and removing the second PEG precipitation step. Both of these steps result in significant loss of yield in the old process.

The development work was started at bench scale with <0.5 kg of suspended Fraction IV-1. After defining the precipitation, incubation, and chromatography steps, the process was increased to macrobench scale (40 kg suspended IV-1) to further evaluate the precipitation, chromatography and ultrafiltration and nanofiltration steps. The entire process was increased to pilot scale (~300 kg PEG filtrate) for reproducibility and toxicology lots.

The scaled up process has reproducibly produced a product that meets or exceeds the purity and viral inactivation goals, and substantially increases the yield of A1-PI. Figure 3 shows a summary of the product produced by the new process. Purity as measured by immunonephelometry and CZE is significantly improved, better than 99% A1-PI. The product is more the 98% monomer as shown by HPLC. In addition the level of pathogen safety is significantly improved by the addition of detergent treatment and nanofiltration. Finally the toxicology scale lots demonstrated a significant yield increase.

We have shown that an improved process for the purification of Alpha-1 PI from Cohn Fraction IV-1 Paste based on the current Prolastin is possible. This new process, Alpha-C improves:
1) Purity to > than 95% monomeric Alpha-1 PI
2) Pathogen safety by adding nanofiltration and a detergent inactivation of enveloped viruses
3) Yield by up to 40%.
Large-scale manufacture of alpha-1-acid glycoprotein for clinical use
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Bio Products Laboratory, Dagger Lane, Elstree, Hertfordshire. WD6 3BX.

Alpha-1-acid glycoprotein (AAG) is a single peptide chain of 183 amino acids and molecular weight of 36 kD. It is a positive acute phase reactant present in human plasma at a concentration of 0.7 to 0.9 g/l, with the level rising 2–4 fold during the acute phase. It has one of the lowest isoelectric points among plasma proteins at 2.7–3.8 and a very high carbohydrate content (~40%) with a large number of sialyl residues. AAG has been reported to have an immunomodulatory activity (1), a stabilizing effect on vascular permeability (2), anti-inflammatory properties (3), and is extensively cited as binding to a range of neutral and basic ligands (4). It was this latter property that provoked an interest in the development of a therapeutic product for treatment of acute drug toxicity.

Several methods have been described for the purification of AAG from plasma and plasma fractions. Some of these are claimed to be suitable for large-scale operation (5). In our experience, none of them were ideally suited to the manufacture of a therapeutic grade AAG at large-scale, and they were considered to intrude into the recovery of other routine products from plasma. The manufacturing process was therefore designed with the following considerations:

- Minimal impact on the manufacture of existing licensed products
- High purity product from a high yielding process
- Ability to operate at "large-scale"
- Incorporation of two independent viral reduction stages in-line with current regulatory requirements
- A stable final product suitable for rapid intravenous administration.

BPL employs modified Kistler & Nitschmann (6) ethanolic fractionation to generate primary plasma fractions for further processing. A screen of the ethanol plasma fractions revealed that the majority of the plasma-derived AAG was present in Fraction V supernatant (FrVs/n) (Table 1). This fraction was therefore selected as the process specific feedstock, and being a side fraction of albumin manufacture it did not affect any other existing manufactured product. FrVs/n contains 40% v/v ethanol, has a pH of 4.8 and is produced at -8 °C. The bulk fraction requires maintenance at <0 °C to ensure protein stability prior to purification. Due to the combination of high ethanol and low temperature, the viscosity of the supernatant is >5 cP, which can be problematic during the subsequent chromatographic stage. In addition to the AAG, FrVs/n contains trace amounts of albumin, alpha-2-HS glycoprotein and a significant amount of fragmented proteins resulting from preceding fractionation stages.

Table 1. Distribution of AAG in plasma fractions from cold ethanol fractionation. *1 liter of the plasma pool undergoing ethanol fractionation generates the respective volumes of supernatant fractions.

<table>
<thead>
<tr>
<th>Fractionation stage</th>
<th>Relative volume*</th>
<th>Yield g AAG/liter plasma</th>
<th>Distribution %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma pool</td>
<td>1.00</td>
<td>0.58</td>
<td>100</td>
</tr>
<tr>
<td>A+1 supernatant</td>
<td>1.19</td>
<td>0.50</td>
<td>86</td>
</tr>
<tr>
<td>Fraction IV supernatant</td>
<td>1.50</td>
<td>0.45</td>
<td>78</td>
</tr>
<tr>
<td>Fraction V precipitate</td>
<td>-</td>
<td>0.04</td>
<td>8</td>
</tr>
<tr>
<td>Fraction V supernatant</td>
<td>1.50</td>
<td>0.47</td>
<td>80</td>
</tr>
</tbody>
</table>
**Purification process**

Fraction V supernatant manufactured by cryoprecipitation and ethanol fractionation of plasma is depth filtered at -5 °C to remove any particulate matter generated from the fractionation process. AAG is isolated from the ethanolic supernatant by anion exchange axial flow column chromatography, using Q Sepharose™ Big Beads (Amersham Biosciences). A 60 column volume load can be applied to the column at 250 cm/h giving a dynamic binding capacity of approximately 15 mg/ml with minimal breakthrough. The use of Q Sepharose Big Beads allows the direct capture and purification of AAG in a single step without the need for any adjustment to the feedstock other than temperature, which is increased to 12 °C using an in-line heat exchanger to reduce the relatively high viscosity. The 200 µm nominal bead diameter is also beneficial in maintaining a low-pressure system with the viscous feedstock. The column lifetime is in excess of 100 cycles, which includes sanitization and storage between cycles in sodium hydroxide.

AAG is eluted from the chromatography medium with a buffer containing 0.2 M sodium chloride, yielding 100% pure alpha-1-acid glycoprotein (Fig 1) at approximately 10 g/l which is >99% monomeric and capable of being stored in a liquid state whilst maintaining excellent integrity and functionality. The purified AAG is batch adsorbed with Aerosil 380 (Degussa). The Aerosil is subsequently removed by depth filtration. The resultant filtrate is concentrated to approximately 100 g/l by ultrafiltration, then diafiltered against 5 volumes of phosphate buffered saline, pH 7.5. The diafiltered concentrate is passed through a 0.1 µm filter prior to nanofiltration.

As a primary virus clearance step, the diafiltered concentrate is passed through a Planova 15N hollow fibre unit (Asahi Kasei) with a mean flux ~10 l/m²/h. Virus filtration results in >6.1 and >5.7 log reduction of polio-1 and hepatitis A viruses, respectively. The inherent stability of the AAG liquid product allows pasteurization of the filled product at 60 °C for 10 hours. At the secondary (terminal) viral inactivation step, pasteurization gives >6 log reduction of polio-1 and vaccinia within 5 minutes and >4 log reduction of hepatitis A. The molecular integrity (Fig 2) and functionality of the product are maintained.

The technology employed for AAG manufacture at pilot-scale is conventional within the plasma processing and biotechnology industry. Each of the unit operations has a proven track record and is particularly suitable for scale-up. It is reasonable to envisage that the process scale could be increased to accommodate batches of several thousands liters of Fraction V supernatant generated by plasma fractionators. A high yielding and scalable production method (Fig 3) which complies with regulatory guidelines and does not compromise the manufacture of any other commonly recovered plasma protein has been developed for AAG, which is currently undergoing clinical trials.

![Fig 1. 1% agarose electrophoresis. Fraction V supernatant (1,3) displays a characteristic 3 band pattern of albumin, alpha-1-acid glycoprotein and alpha-2-HS glycoprotein (anode to cathode respectively), for which the identity is confirmed by immunoelectrophoresis (5,7,9). Fraction V supernatant anion exchange flow through (4) is much reduced in alpha-1-acid glycoprotein indicating the successful direct adsorption of AAG on to the anion exchange medium. Anion exchange flow through (6,8) shows the presence of the three main protein components. Salt elution of the anion exchanger yields a “pure” (single band) AAG product (2).](image1)

![Fig 2. Comparison of molecular weight distribution between unpasteurized and pasteurized AAG. HPLC TSK3000 size exclusion chromatography: unpasteurized AAG (1), pasteurized final product (2).](image2)
Fig 3. AAG manufacturing process flow sheet.

Acknowledgements
Our thanks go to Dr. Peter Roberts and the R&D Virology group for providing data on viral reduction.

References
Intravenously applied IgG stimulates complement attenuation in dermatomyositis at the amplifying C3 convertase level

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Introduction

Intravenously applied human IgG (IVIG) is beneficial in autoimmune diseases that are associated with excessive complement activation via the classical pathway and the amplification loop (1,2). Such complement activation is pro-inflammatory by generating C3a and C3b in excess. High-dose IVIG suppressed complement-dependent tissue destruction in dermatomyositis by displacing nascent C3b from tissue-bound immune complexes to fluid phase IgG (3). IgG is a preferred target of nascent C3b and forms C3b-IgG complexes (4). These complexes are 7–10 times more efficient activators of the alternative complement pathway than immobilized C3b (5,6). Thus, if displacement of nascent C3b to fluid phase IgG were the only complement-modulating effect of IVIG, complement amplification would have continued in the plasma of patients with dermatomyositis, which it evidently did not. We therefore studied the effect of high-dose IgG on complement amplification. High-dose IgG had an attenuating effect on complement amplification in vitro; IgG concentrations comparable to those reached during an IVIG treatment lowered the half life of C3b-IgG complexes in 20% serum from 3–4 to 1–2 min (4). Here we report that high-dose IVIG attenuated complement amplification in vivo, and stimulated inactivation of C3b-contained complexes (Blood, first edition September 2003).

Results and discussion

High-dose IVIG (2 g per kg body weight) activated the classical complement pathway in patients with dermatomyositis with a 30–40% consumption of C4 during the first 3–4 days. Surprisingly, in parallel, the C3 concentration remained constant in myopathic and increased significantly by 15–20% in amyopathic dermatomyositis. This implies that a high-dose IVIG treatment transiently activated the classical complement pathway, but at the same time attenuated complement amplification, whereas the C3 concentration remained at initial values or increased slightly.

Attenuation of complement amplification by IVIG suggested that it may interfere with C3 convertase precursors. To address this question, we studied the concentration of the short-lived C3b-containing complexes in patients’ plasma. C3b-containing complexes were visualized/identified by a monoclonal antibody (H206) that detected the presence of C3b, but not inactivated C3b (iC3b). In the course of the first IVIG treatment of dermatomyositis patients, we noticed the following changes in the level of C3b2-containing complexes:

1) During infusion of up to 25% of total IVIG, which corresponded to an infused dose of 0.5 g/kg b.w., the C3b2-containing complexes dropped to 37 ± 14% (n=6) of the pretreatment level.
2) Beyond that dose until full dose was applied, C3b2-containing complexes increased marginally and in parallel to factor Bb, which did not exceed 1.5 times the physiological level.
3) Thereafter the concentration of C3b2-containing complexes decreased and reached by day 14 66 ± 19% (n=4).
4) By day 28–30 the concentrations of these complexes were no longer significantly different from pretreatment levels.
Thus, IVIG attenuated complement amplification *in vivo* sufficiently to stop excess C3 consumption that could have resulted from transient activation of the classical pathway. If IVIG did not contain substances that stimulated inactivation of C3 convertase precursors, the dose of 2 g/kg b.w. would have substantially raised factor Bb concentration and caused a C3 consumption at least proportional to that of C4. This would have resulted in an estimated decrease of C3 by 10–15% (based on the relative concentrations of C4 and C3). Instead, we found a 15–20% increase in C3 in amyopathic and a constant level of C3 in myopathic patients. The attenuating effect of IVIG on complement amplification further compensated for the extra amounts of C3 activated by IVIG through the classical pathway. The potential of IVIG to attenuate complement amplification appears crucial because it might help to prevent immune-complex like diseases induced by interactions of IVIG with endogenous immunoglobulins in IVIG-treated patients.

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Lisy of posters presented at PPB 03, Curaçao, Netherlands Antilles

*Denotes author for correspondence

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   1. Amersham Biosciences, SE 75184 Uppsala, Sweden
   2. Department of Molecular and Structural Biology, University of Aarhus, Denmark

2. Recovery of IgG from ethanol precipitates of the Cohn-Oncley and Kistler-Nitschmann fractionation schemes
   John Curling, Dev Baines, Crystal Russell, Keith Watson, Emma Ward, Hannah Pollard and Steve Burton
   ProMetic BioSciences Ltd., 211 Cambridge Science Park, Cambridge CB4 OZA, UK

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   Tatiana V. Grinenko, Georgiy L. Volkov, Olena S. Havriliuk, Olena I. Yusova, Marina B. Zadorozhnaya
   Palladin Institute of Biochemistry of the NAS of Ukraine
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   V. Gurevich, K. McCann, K. Lyons, J. Bertolini
   CSL Bioplasma, 189-209 Camp Rd, Broadmeadows, Victoria, 3047, Australia

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   K. McCann, P. Gomme, L. Fernandez, B. Hunt, J. Bertolini
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   Peter Gomme, Sharon Vyas, Liza Fernandez, David Eakins, Joe Bertolini
   CSL Bioplasma 189-209 Camp Road, Broadmeadows, Victoria 3047, Australia

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   A. Johnston, P. Gomme, O. Tatford, D. Dunstan, J. Bertolini
   1. CSL Bioplasma 189-209 Camp Road, Broadmeadows, Victoria, Australia, 30471
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8. "Size exclusion filtration to remove TSE infectious particles from a model protein stream using composite membrane"
   Vincent Pizzi, Robert Blanck, Christopher Stenland, Jarrett Terry, Kang Cai, Richard Rubenstein, Michel Fourne, Douglas C. Lee, Stephen Petteway
   1. Millipore Corp.
   2. Bayer Corp.
   3. Dept. of Virology NY, State Institute for Basic Research in Developmental Disabilities

9. Storage study performed on immunoglobulin G in solution
   I. Andersson, L-O. Lindquist, C. Sund Lundström
   Amersham Biosciences, Uppsala, Sweden

10. Continuing production of a chromatographic purified strategic product – human albumin 5% over 20 years
    Olga Damevsk Todorovska, P. Kolevski, V. Stefanovska
    Institute of Blood Transfusion, Medical Faculty, University “St. Cyrilus and Methodius” 1000 Skopje, Vodnjanska 17 Republic of Macedonia.
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Center of Genetic Engineering and Biotechnology, 31 Avenue between 158 and 190 streets, PO Box 6162, Cubanacán Playa, Ciudad de la Habana, Cuba. PC 10600

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Dr, T. Zandieh, Dr, M. Farhadi, Dr, M. Shahrabadi
Iranian Blood Fractionation & Research Co, Tehran

13. INACTINE™ PEN110 and solvent/detergent: Two orthogonal virus inactivation methods
John Chapman*, Asa Ohagen, Veronica Gibaja, Jennifer Marcello, Douglas Lunderville, Jett Horrigan and Aris Lazo
V.I. Technologies, Inc., Watertown, MA 02472, USA

14. A study of the purification process of Nanotiv (factor IX)
Anna Mjärdestam, Peter Aizawa, Robert Marschall, Christina Leo, Stefan Winge
Octapharma AB, Stockholm, Sweden

15. Parameters important for the effective usage of a nanofiltration step
Stefan Winge
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16. Use of Llama antibody fragments as ligands in affinity chromatography
Pim Hermans*
Biotechnology Application Centre BV, The Netherlands

17. Methods for the removal of INACTINE™ and analysis of INACTINE at trace level concentrations
Inger Andersson, Stefan Eriksson*, Ola Lind and Peder Bergvall
Amersham Biosciences, Separations R&D, SE-75184 Uppsala, Sweden

18. iProMx™: a new concept in the control and monitoring of production processes
Brendan J. Murray¹, Daniel Liebhart², and Neil H. Goss*³
¹ Avalis AG
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19. Intravenously applied IgG stimulates complement attenuation in dermatomyositis at the amplifying C3 convertase level
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20. Characterization of recombinant human serum albumin using ettan MALDI-ToF Pro
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