More than a century after the first successful use of serotherapy, antibody-based therapy has been renewed by the availability of recombinant monoclonal antibodies. As in the past, current clinical experience has prompted new pharmacological questions and induced much debate among practitioners, notably in the field of ophthalmology.

An examination of the history of antibodies as treatments for ocular disorders reveals interesting parallels to the modern era. The fact that a treatment administered by a systemic route could be efficacious in a local disease was not widely accepted and the “chemical” nature of antibodies was not clearly understood in the late 19th century. Clinical studies by Henry Coppez, a Belgian ophthalmologist, established in 1894 that antipolyvalent antitoxins could be used to treat conjunctival diphtheria. Nearly 20 years later, Coppez and Danis described age-related macular degeneration, a disorder which today benefits from ranibizumab therapy. The product, a locally-administered recombinant Fab fragment, is directed against vascular endothelial growth factor A. Interestingly, its full-size counterpart, bevacizumab, which is approved for the treatment of solid tumors, has also demonstrated efficacy in “age-related” macular degeneration when administered either intravenously or locally, which raises new questions about antibody pharmacology and biodistribution.

In order to shed some light on this debate, we recount the early history of serotherapy applied to ophthalmology, review the exact molecular differences between ranibizumab and bevacizumab, and discuss what is known about IgG and the blood-retina barrier and the possible role of FcRn, an IgG transporter.

Historical Perspective

The recent emergence of anti-vascular endothelial growth factor (VEGF) antibodies for the treatment of age-related macular degeneration (AMD) revives an old tradition of successful serotherapy for ocular diseases. In the 19th century, conjunctival diphtheria was an uncommon form of ocular disease, but the infection was particularly severe in children and could cause eye loss. The infectious agent, the Löffler-Klebs bacillus (Corynebacterium diphteriae) and its toxin induced a profuse exudation in the conjunctivae that tended to coagulate, leading to necrosis of infiltrated tissues.

In February 1894, Roux, Martin and Chaillou observed that the recently discovered antidiaphtheritic serum used to treat croup also cured its ocular manifestations. Later that year, Henri Coppez, a Belgian ophthalmologist (Fig. 1), was the first to use anti-diphtheritic antitoxins to treat conjunctival diphtheria in two young children, and he concluded that clinical results were spectacular: “Sous l’effet de l’injection, les fausses membranes semblent fondre comme un flocon de neige dans un rayon de soleil” translated as “Under the effect of injection, the false membranes seem to melt like a snow flake in a ray of sunshine.” This new treatment rapidly spread through-out Europe and beyond,
e.g., Italy,5 England,3 Germany6 and Russia.7 All initial case reports described therapeutic successes.4-10

The first “clinical trial” using the new therapy was published in 1895 by Ernest Aubineau, a French ophthalmologist (Fig. 2). At the time, double-blind placebo-controlled trials were not standard and so a noncomparative study in ten consecutive children (newborn to 8 years) who suffered from conjunctival diphtheria as demonstrated by the presence of Löffler bacillus was performed.11 Clinical efficacy was observed in all patients, with regression consistently occurring 24–48 hours after treatment. This success even prompted Aubineau to propose anti-diphtheritic serotherapy as a diagnostic test of diphtheria in infectious conjunctivitis. The results are all the more remarkable because different sources and preparations of anti-diphtheritic antitoxins were used, with inevitable variations in terms of quality and quantity (antitoxic titer). In the following years, the worldwide needs of serum were met by an increase in the number of “bacteriology institutes” that prepared anti-diphtheritic sera according to several methods.

Serotherapy of ocular diseases was also revolutionary because antitoxins were administered through a systemic route, whereas ocular treatments were traditionally applied locally. Indeed, Aubineau11 and Coppez13 used, respectively, subcutaneous and intramuscular injections for the treatment of croup. Parents of sick children, as well as the physicians, did not fully understand why a therapeutic effect could be obtained away from the site of the injection.11 The “chemical” nature of the antitoxins was indeed mysterious because nothing was known about immunoglobulins, their half-life and their distribution in the organism at that time.

Coppez suggested that the antitoxin may also be administered locally by intraconjunctival infusions in order to protect the cornea from the deleterious action of toxin;13 however, the immunogenicity of horse serum proteins and immunoglobulins was then also unknown. Interestingly, Coppez noted that certain symptoms, e.g., exanthemas, localised oedemas, arthralgia, became more frequent after serotherapy was introduced and happened thirteen days after the first injection.13 This syndrome was referred to as serum sickness by Clemens von Pirquet and Bela Schick in 1905,14 long before its pathophysiology was understood.

Recombinant Antibodies in Ophthalmology: Ranibizumab and Bevacizumab

In addition to being a pioneer of serotherapy in ophthalmology, Henri Coppez is also linked to modern antibody therapy because he and Marcel Danis were the first to describe exudative senile macular degeneration in 1923.15 This disease, now known as AMD, is currently treated with recombinant antibodies, a modern form of serotherapy. Donald Gass suggested in 196716 that the neovascular proliferation may play a primary role in the pathobiology of the disciform lesion reported in AMD. With the identification of vascular endothelial growth factor A (VEGFA) as a key agent in neovascularization and vascular leakage in AMD,27-18 anti-VEGF “antitoxins” targeting this endogenous “toxin” (or “autoxin”) began to be developed. Polyclonal antibodies of animal origin

Figure 1. Henri Coppez, a history. Henri Coppez was born in Brussels on September 9, 1869 and died in the same city on August 26, 1946. His father, Prof. Jean-Baptiste Coppez (1840–1930) founded the ophthalmology’s rostrum in the Université de Bruxelles where Henri obtained his M.D. degree in 1893. Coppez specialized in ophthalmology in Vienna with Fuchs, in Utrecht with Snellen, and in Brussels with his father, serving as an assistant from 1894 to 1897. He obtained the special doctorate in 1897 with a thesis on the natural history and treatment of pseudomembranous conjunctivitis, with special emphasis to serotherapy. Coppez entered the Brugmann hospital in 1922, succeeded to Gallemaerts in 1925, and was appointed as professor in 1927. When he retired in 1930, he worked in the Institut Coppez that he founded in 1913. Henry Coppez died in 1946 while preparing a speech on the scientific activities in Ophthalmology: Ranibizumab and Bevacizumab

Figure 2. Ernest Aubineau, a history. Ernest Aubineau was born on January 13th, 1871 and died on October 1st, 1951. He began his medical studies in Nantes, France and specialized in ophthalmology, first with Prof. Dianoux in Nantes, and then with Prof. de Wecker in Paris where he discovered the usefulness of serotherapy. In 1895, he obtained his M.D. degree with a thesis entitled “Essai sur l’application de la sérothérapie dans le traitement de la diphtérie conjonctivale.” Although he decided to work in private practice, first in Brest and, from 1913, in his native region, he continued to make substantial scientific endeavors, regularly publishing at the congresses of the French Society of Ophthalmology, of which he was member since 1897, and in journals specializing in ophthalmology.
are no longer necessary because molecular biology and protein engineering techniques can now be used to produce monoclonal antibodies (mAbs) that are largely humanized, but the principle and the goal remain identical.

**Bevacizumab and Ranibizumab Development**

Genentech developed humanized mAbs targeting VEGF, although the initial application was for cancer, not AMD. First, the murine anti-VEGF mAb A.4.6.1 with neutralizing activity on all VEGF isoforms was selected and cloned. The humanization of muMAb VEGF A.4.6.1 consisted of the transfer of the six complementarity determining regions (CDRs) from muMAb VEGF A.4.6.1 to a consensus human framework used in previous humanizations. Several framework residues in the humanized variable heavy (VH) domain and one framework residue in the humanized variable light (VL) domain were changed from human to murine to achieve binding equivalent to muMAb VEGF A.4.6.1. The resulting Fab (Fab-12) was used to synthesize a full-length IgG1κ humanized mAb (rhuMab-VEGF or Fab-12 IgG1) now known as bevacizumab (Avastin®) (K<sub>d</sub> = 433 pM).22,23

Bevacizumab, administered intravenously, was first approved for marketing in 2004 and is currently used in the treatment of certain advanced or metastatic forms of colorectal, breast, lung and kidney cancer. At the time, it was thought that bevacizumab could not be used in the treatment of AMD because comparisons of the intravitreal injection of another full-length antibody (trastuzumab, Herceptin®) with its Fab indicated that only the latter allowed full retinal penetration, whereas the full-length antibody did not penetrate the retina. This was consistent with the observation that molecules larger than 77 kDa do not freely diffuse across fixed human retina.25 These observations led Genentech to develop an anti-VEGF Fab by selection of an affinity-improved variant of Fab-12, rhuFabV2, which is now called ranibizumab (Lucentis®).26 To obtain this increase in binding affinity and allow inhibition of VEGF with its monovalent Fab format, the original Fab was genetically engineered through a process of CDRs random mutagenesis and affinity selection (Fab Y0317 mutant, K<sub>d</sub> = 19.8 pM).23 The resulting ranibizumab (a Fab) differs from the corresponding part in bevacizumab by six amino acids. Five differences are in the variable domains, as exemplified in the IMGT Protein displays (Fig. 3) and by comparison of the IMGT Colliers de Perles of 1cz8 and 1bj1, respectively, available in IMGT/3Dstructure-DB (http://www.imgt.org). Four differences are in the VH, two in the CDR1-IMGT (T29>D, N36>H) and two in the CDR3-IMGT (H109>Y, S112>T), and one difference is in the V-KAPPA domain (M4>L). The sixth difference is in the hinge region (T10>L). Most of the mutations take place in the ranibizumab antigen binding site, i.e., its paratope, according to IMGT unique numbering.27

As a Fab, ranibizumab has a total molecular mass of 48.39 kDa; it is not glycosylated and can be easily produced in E. coli. On the other hand, bevacizumab, which is a full-size IgG1κ, has a molecular mass of 149 kDa, is N-glycosylated in its Fc region, and requires mammalian cell lines CHO DP-12,20 for production. Due to its simpler structure and its higher affinity for VEGF, ranibizumab requires lower molar amounts than bevacizumab to neutralize an equal amount of VEGF.24 Despite preliminary data suggesting that a full-length antibody would not be efficient for intravitreal injections, the earlier commercial availability of bevacizumab compared to ranibizumab led ophthalmologists to try the former as an AMD treatment. As a result, bevacizumab was used by intravitreal injection with success and came to be an attractive alternative because of its lower cost compared to ranibizumab.29 As intravitreal injection of bevacizumab is an off-label indication of AMD treatment, little is known about the product’s pharmacokinetics (PK) in the eye, which needs to be studied before
dosing regimens can be optimized and its safety assessed. Several studies have provided some answers by analysing the PK of Fab or full-length antibodies after their intravitreal injection. 24,26,30,31 Bakri and associates injected bevacizumab 26  or ranibizumab 31 in one of the two eyes of rabbits and measured their concentration in vitreous humor, aqueous humor of the two eyes, and serum. The intravitreal half-life of bevacizumab was estimated at 4.3 days and the antibody was detectable in the serum, as in the uninjected eye, at a very low concentration. Although Mordenti and associates studied another full-length antibody, trastuzumab, with another protocol in another animal model, they reported a similar half-life of 5.6 days in the eyes of monkeys. 24 PK studies of intravitreal ranibizumab reported a half-life of 2.6 days 26 (monkey) or 2.88 days 31 (rabbit). The Fab was detected in the serum of monkeys, but not in serum or uninjected eyes of rabbits.

Although the systemic route of administration was less studied in AMD, efficacy of bevacizumab was reported and authors suggested that this more comfortable route of administration may be used for AMD treatment. 32 Unsurprisingly, the question of how a full-length antibody crosses the blood-retina barrier was discussed. In 2003, Saishin and associates showed that the subcutaneous administration of a fusion protein comprising ligand binding elements taken from the extracellular domains of VEGF receptors 1 (FLT1) and 2 (KDR) fused to the Fc portion of IgG1 (VEGF-TRAP RIR2 or aflibercept) significantly inhibited subretinal neovascularization in transgenic mice in which express VEGF in photoreceptors. VEGF-TRAP RIR2 reduced the breakdown of the blood-retina barrier in two such models of VEGF-induced rupture. 33 These observations are consistent with the presence of neonatal Fc receptor (FcRn) in rat eyes and particularly on the blood-retina barrier. 34 FcRn is known to modulate IgG transport and protect against their catabolism, resulting in their long serum half-life as compared with other proteins. The presence of FcRn may explain why VEGF-TRAP RIR2 was able to cross the blood-retina barrier, i.e., its IgG1 Fc portion interacted with FcRn. Such a mechanism may also explain why bevacizumab, an IgG1, is efficient in AMD when administered by a systemic route.

**Conclusion**

One century apart, the story of serotherapy in conjunctival diphtheria and that of anti-VEGF mAbs in AMD show important similarities, particularly due to the fact that clinical experience, which remains in a large part empirical, raises many questions about the pharmacology of therapeutic antibodies. It is also increasingly clear that the pre-clinical in vivo models are not necessarily relevant to the human situation, particularly for antibodies that are now largely humanized or human. This is probably the reason why the clinical experience sometimes breaks established dogmas, and sends researchers back to the bench. This is particularly true concerning IgG and protein trafficking across the blood-retina barrier.

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