Use of colloidal gold surface plasmon resonance peak shift to infer affinity constants from the interactions between protein antigens and antibodies specific for single or multiple epitopes

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The surface plasmon resonance (SPR) wavelength of colloidal gold particles coated with a monoclonal antibody is red-shifted when the antibody interacts with its specific ligand. This shift results from the change in the refractive index of the particles as induced by ligand binding. This property is used to monitor in real-time the association and dissociation kinetics of the interaction in solution. The monitoring is perfomed in a clinical chemistry automated analyzer during a few minutes of incubation at 37 °C. Data treatment allows calculation of the affinity constant of the interaction. The SPR wavelength shift does not necessarily require agglutination or aggregation of the particles to occur since particles coated with one monoclonal antibody specific for a single epitope on the ligand can be used in the procedure. The affinity constants measured by this procedure correlate with those calculated from Scatchard plots or BIAcore data.

Keywords: *Gold surface plasmon resonance; surface plasmon absorption spectrometry; colloidal gold particle immunoassay; clinical chemistry analyzer; antibody affinity constant; protein antigens*

Direct immunosensor devices (*i.e.*, homogeneous immunoassays) are comprised of antibody species coupled to a signal transducer detecting the binding of the antigen by a change in potential difference, current, resistance, mass, heat, or optical properties.[1] Chemical materials able to serve as transducers in such devices are currently hot topics in immunochemical research. These last years, we have been particularly keen to apply the optical properties of conducting polymers[2] and of colloidal gold to homogeneous immunoassays.

Gold colloids can be easily synthesized in sizes ranging from 2 to 300 nm diameter. The sols are characterized by a discrete band in the visible electronic spectrum called the surface plasmon resonance (SPR). The surface plasmon is a quantized plasma oscillation occurring at the surface of the gold particle resulting in a characteristic peak location at maximal absorbance (A) and peak width.[3] The SPR wavelength and width depend on the particle diameter[3] and any increase in the average diameter of the particles in a given sol induces a red shift of the SPR wavelength along with a decrease in maximal A. This is the reason why a sol characterized by interparticle distances greater than the average particle diameter appears red and when the interparticle distance in aggregates decreases to less than approximately the average particle diameter, the sol color changes to blue.[4]

The remarkable colorimetric properties of colloidal gold have made it possible to use it for many years as reporter reagent in sandwich enzyme immunoassays[5] and more recently in the selective colorimetric detection of complementary polynucleotide strands.[4, 6] In all these applications, however, it was considered that either the specific agglutination or aggregation of the DNA- or antibody-coated particles had to occur for a significant signal to be recorded.[4, 7] Recently, monolayers of colloidal gold particles have been successfully coated on quartz or glass plates with the aim of serving as a basis for biosensing devices.[8]

For some time, we have successfully applied the characteristic features of colloidal gold SPR to quantitative short incubation homogeneous sandwich immunoassays performed in automated clinical chemistry analyzers. During a feasibility study conducted for Hoffmann-La Roche, we noted that a measurable SPR shift could be observed when colloidal gold particles of a suitable size coated with individual well characterized monoclonal antibodies specific for a single epitope were interacting with the ligand. This observation meant that neither agglutination nor aggregation of the coated particles were required for the SPR shift to occur, and that the subtle particle mass increase occurring upon ligand-binding was likely to be of sufficient importance so as to induce a recordable change in the colour of the colloidal solution.

SPR detection on coated gold films is currently widely used in sophisticated and expensive instruments such as the BIAcore system commercialized by BIAcore AB (Uppsala, Sweden) for monitoring biomolecular interactions in real time.[9, 10] Further to our observation, we report that SPR shift detection by an automated photometer in liquid phase can be used for the same purpose. The major advantages of this potential new application when compared to the BIAcore technology result from the ease of coating the colloidal gold reagent with antibodies and from its use in cheaper automated clinical chemistry analyzers.

Experimental

Reagents

Tetrachloroauric acid hydrate, poly(ethyleneglycol) 6000 (PEG) and anhydrous citric acid were obtained from Acros Organics (Geel, Belgium). Bovine serum albumin (BSA) was purchased from Calbiochem (San Diego, CA, USA). All other chemicals and buffers used were of the highest grade available from Aldrich (Bornem, Belgium).

The anti-human heart fatty acid-binding protein (hFABP) monoclonal antibodies and recombinant hFABP were kindly supplied by Hoffmann-La Roche (Basel, Switzerland). The full characterization of the antibodies with the BIAcore system has been reported.[11] The anti-human chorionic gonadotropin (hCG) and anti-human ferritin (hFer) monoclonal antibodies were purchased from OEM Concepts (Toms River, NJ, USA). Their specificity and affinity characteristics calculated from Scatchard plots were provided by the manufacturer. All the antibodies used in this study were affinity-purified. Pure hCG and liver hFer were purchased from Scipac Ltd (Sittingbourne, UK). The ligands were dissolved in 0.15 M saline. The incubation buffer

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used throughout was 0.2 M TRIS-HCl pH 8.0 containing 50 mM of NaCl, 10 mM EDTA, and 70 g l^{-1} PEG.

Colloidal gold

Colloidal gold was synthesized by reduction of a hydrogen tetrachloroaurate aqueous solution by sodium citrate at ebullition as previously reported.[12] The relationships between average particle diameter or heterodispersity as measured by electron microscopy and either SPR wavelength or width, respectively, were established according to data reported in the literature.[13] The SPR width was expressed as the ratio of *A* at 600 nm over maximal *A*. The number of particles per unit volume of reagent was calculated according to published relationships with the maximal *A* of the sol.[14]

Colloidal gold was coated with the various antibodies by charge adsorption according to published procedures.[15, 16] In a typical process, the antibody is diluted with water at a concentration of 50 μg ml^{-1} and the gold sol is adjusted to pH 8.5 with NaOH. Whilst stirring the colloid vigorously, a sufficient quantity of the antibody solution is rapidly added so as to cover entirely the particles present in the sol and thereby stabilize them against coagulation by salts. The mixture is then incubated for 10 min at room temperature and the sol is further buffered with 0.1 M borate, pH 8.0, and stabilized by the addition of BSA (5 g l^{-1}) and of Tween 20 (0.2% v/v).

Equipment and data treatment

The incubations and kinetic measurements were performed in a Cobas-Mira Plus automated clinical chemistry analyzer kindly provided by Hoffmann-La Roche (Basel, Switzerland). Absorbance at 600 nm was recorded every 25 s during incubation at 37 °C. The raw *A* data were mathematically treated for blank subtraction and linearization. In a typical experiment, 30 μl of sample (either containing the ligand or devoid of ligand for the blank measurement) were preincubated during 2.5 min with 150 μl of buffer so as to subtract any possible sample blank. The antibody-coated gold colloid (50 μl) was added and the mixture further incubated during 10 min. An excess ligand (50 to 400 fold in 2 μl) was then added in order to induce the dissociation that was followed up during a further incubation of 8.3 min.

The visible spectra were recorded in a double-beam Lambda 2 spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). Spectra were recorded after 10 min of incubation at room temperature of mixtures made of 100 μl of sample in the absence or presence of the ligand, 500 μl of buffer and 200 μl of antibody-coated colloidal gold.

Results and discussion

Application of colloidal gold to automated quantitative immunoassays

Because of its remarkable colorimetric properties, colloidal gold has been used for many years as reporter reagent in sandwich immunoassays[5, 17] and detailed techniques have been published for the quantitative detection of polypeptide antigens such as hCG,[18, 19] antibodies to the human immunodeficiency virus[20] or antibodies to cell surface antigens.[21] Gold colloids have also been used in homogeneous competitive agglutination inhibition assays for steroid ligands such as estrogens.[22] These assays were designed on the sandwich enzyme immunoassay principle and required a rather long incubation period before the colorimetric signal due to the gold–antibody conjugate agglutination could be measured.[7] The authors preferred also to measure the decrease in *A* at the SPR (540 nm) rather than to record the increase in *A* at longer wavelengths resulting from the SPR shift.

During the last few years, we have successfully applied the principle of SPR shift in homogeneous immunoassays run in clinical chemistry automated analyzers. Because of the low incubation time allowed by such instruments (maximum 20.8 min), the kinetics of sandwich formation was optimized and speeded up by the addition of PEG as an enhancer in the incubation buffer. Furthermore, we found that recording *A* at longer wavelengths rather than at the SPR improved the sensitivity. The size of the gold particles to be used in such assays was optimized by varying the citrate–gold ratio during synthesis and it was found that particles with an average diameter around 40 nm were suitable for such an application. These allow for a SPR shift to occur which induces a significant *A* increase at 600 nm, a wavelength for the light measurement of which most automated analyzers are equipped. During the course of a feasibility study conducted for Hoffmann-La Roche and aimed at applying these optimized conditions to the quantitative determination of hFABP on the Cobas-Mira plus, we tested independently colloidal gold coated with identical concentrations of three different monoclonals, namely 53E9, 67D3, and 72H10 over a wide range of hFABP concentrations. The dose-response relationships obtained from these experiments are shown in Fig. 1. The observation of such relationships was quite surprising because the three antibodies had been fully characterized with the BIAcore and had been definitely shown to recognize each a single independent epitope on the hFABP molecule.[11] The common consideration, as backed by data from the literature,[4, 7] is that agglutination or aggregation of the colloid is required for a significant SPR shift signal to be recorded. In the present case, neither agglutination nor aggregation could explain the signal because each antibody was specific for a single independent epitope. Consequently, the signal resulting from the SPR shift could only originate from a change in the refractive index of the individual particles subsequent to

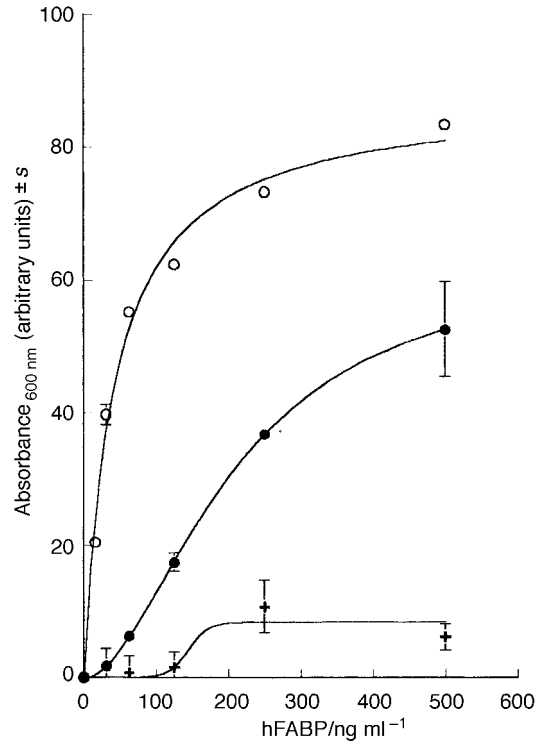

Fig. 1 Dose–response curves observed after incubation in the Cobas-Mira in duplicate during 20 min of mixtures containing a fixed amount of colloidal gold reagents independently coated with the three monoclonal anti-hFABP antibodies (72H10, crosses; and 67D3, closed circles; 53E9, open circles) and samples containing increasing concentrations of hFABP. The antibodies recognize each a single distinct epitope on hFABP. Consequently, the dose–response curves observed cannot be explained by the agglutination of the colloid.

a further coating by the ligand when binding to the antibody. The dose–response curves shown in Fig. 1 revealed also another interesting feature which was likely to support the hypothesis, *i.e.*, the inverse relationship between the height of analytical response and the affinity of the respective antibodies as measured in the BIAcore system.[11] Upon binding an identical ligand dose, the antibody with the highest affinity (72H10) provided the lowest response and the antibody showing the lowest affinity (53E9) displayed the highest response, whilst the response of the antibody with an intermediate affinity (67D3) was in between. Because the same quantity of each individual antibody was coated on the particles, this behaviour was consistent with the inversely proportional relationship between antibody affinity and binding capacity.[23]

Origin of the SPR shift

In order to examine the phenomenon more in depth, we recorded the visible spectra of mixtures containing colloidal gold, respectively coated with various monoclonal antibodies after incubation with increasing doses of the respective antigen. As an example, Fig. 2 shows the SPR shift and concomitant decrease in maximal *A* of a fixed quantity of colloidal gold coated with a monoclonal antibody (M2F03) specific for the hCG α-β subunits joining epitope,[24] as a function of the increasing hCG amounts with which it has interacted. SPR is a function of the effective refractive index of the metal surface which is determined by the mass of the substances present at the interface.[25] Consequently, the shift in SPR wavelength is supposed to reflect small changes in the refractive index at the particle surface which are the direct result of mass changes in the approximate medium. When spherical gold particles are used such as in a colloidal solution, the SPR wavelength is directly related to the diameter of the particles.[3] With the aim of verifying this mechanism of wavelength shift, we calculated the average diameters of the particles before and after interaction with the ligand. From these data, we were able to infer the apparent changes in volume of the particles and depending on the antibody specificity, to calculate either the number of particles present in the agglutinates, or the number of molecules of ligand present at the surface of individual particles, respectively (protein molecules were considered as spheres and the volume of individual molecules was calculated from their Stokes radii). When the antibodies coating the colloidal gold particles were not specific for a single epitope, the increase in particle volume observed after interaction with the ligand resulted from particle agglutination as shown in Fig. 3 for two monoclonals against hFer. Counter to that, when the antibodies were specific for a single epitope on the ligand, the increases in particle volume observed after incubation with the ligand were too small for coping with the presence of additional particles and could be explained only by the presence of ligand molecules bound on the surface of the antibody-coated particles. Such case is illustrated in Fig. 4, for two monoclonal antibodies specific for a single epitope of hCG. It is worth noting that in this latter case, the calculated number of hCG molecules bound are in agreement with data reported in the literature for ligands of similar molecular weight adsorbed in a non-specific way on the surface of colloidal gold particles.[26]

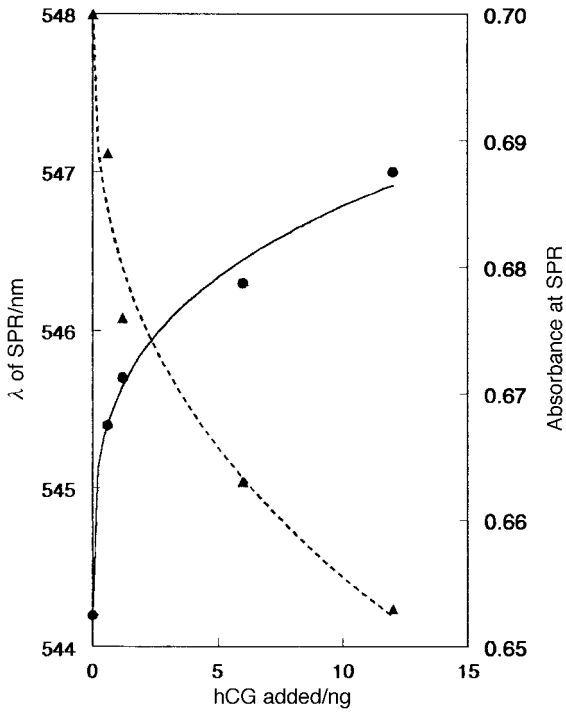

Fig. 2 Shift of the SPR (closed circles, solid curve, left axis) and concomitant maximal *A* decrease (closed triangles, dotted curve, right axis) of colloidal gold particles coated with a monoclonal anti-hCG antibody (M2F03), calculated from visible spectra recorded after incubation of a fixed amount of the colloidal gold reagent with samples containing increasing concentrations of hCG. The antibody is specific for the α-β subunits joining epitope on the molecule.

Application of colloidal gold SPR shift to the determination of affinity parameters from biomolecular interactions in a clinical chemistry automated analyzer

Clinical chemistry automated analyzers are walk-away instruments capable of mixing and incubating reagents and samples in individual cuvettes according to programmed conditions. Such instruments are further equipped with a flash lamp photometer and are consequently able to monitor subtle changes in *A* in each cuvette at a given wavelength and at short regular periods of time.[27] These instruments are widely used in clinical laboratories for the quantitative analysis of blood and urine samples. In this study, we used a Cobas-Mira Plus instrument. This instrument is capable of performing incubations at 37 °C during up to 20.8 min and to record *A* changes in individual cuvettes every 25 s. Such an instrument is able to handle data of up to 120 samples per hour.

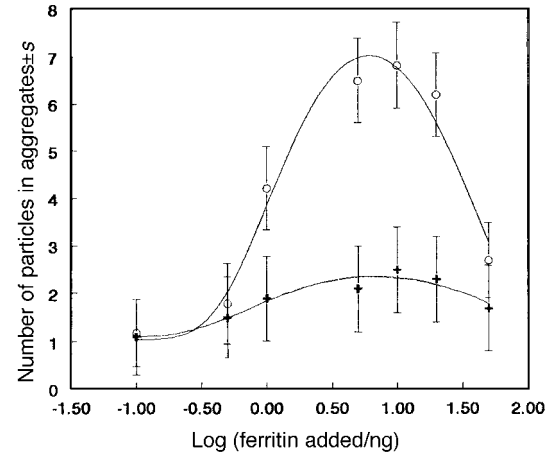

Fig. 3 Relationship between the calculated number of particles present in clusters formed after incubation of colloidal gold coated, respectively, with two anti-hFer monoclonals (057-10030, crosses; and 090-10175, open circles) and the log of ligand amount added in the mixture. The number of particles was inferred from the difference in volume observed after incubation in presence of the ligand. The antibodies are not single-epitope specific.

Further to the observation of SPR shift we had made with colloidal gold particles coated with various antibodies, including monoclonals specific for a single epitope on the ligand molecule, we attempted to apply the principle to the real-time monitoring of association and dissociation kinetics in the automated instrument. The instrument was programmed so that the colloidal gold–antibody conjugate was added to mixtures containing ligand solutions of increasing concentrations and incubation buffer, and the association reaction was monitored by the *A* increase at 600 nm during 10 min. A second ligand solution containing a several fold excess was then added and the dissociation was further monitored by the *A* decrease at 600 nm during 8.3 min. Examples of association and dissociation kinetics are shown in Fig. 5. The colloidal gold particles were coated with the monoclonal anti-hFABP 53E9 and incubated with samples containing four increasing ligand concentrations. This antibody recognizes a single epitope specific to the human heart protein as it does not cross-react with the protein from human intestine, human liver or bovine heart despite the high level of sequence homology (87%).[11] Table 1 lists the comparison of the apparent affinity constants calculated from the association and dissociation rate constants of kinetics observed for ten antibodies coated on colloidal gold, with the apparent affinity constants obtained from Scatchard plots or the BIAcore instrument, respectively. The coefficient of correlation between the apparent affinity constants obtained by the method reported here and those obtained by the two latter are, respectively, 0.96 and 0.98. The apparent affinities obtained by the colloidal gold method are in good agreement with those calculated from Scatchard plots. Despite the good correlation, their level of importance differ, however, significantly from those calculated with the BIAcore system, a discrepancy already mentioned for relative affinities obtained from enzyme immunoassay.[11]

This report shows that the SPR shift of colloidal gold particles coated with antibodies of various specificities occurring upon interaction with the ligand can be applied to the measurement of apparent affinity constants in automated clinical chemistry analyzers. This technique has undoubtedly a potential for the high throughput screening of combinatorial chemistry libraries. The automated instrument along with the reagents are much cheaper than those used in more sophisticated and expensive biosensors. We have shown that the technique was suitable for the measurement of apparent affinities between antibodies and protein ligands, the smallest

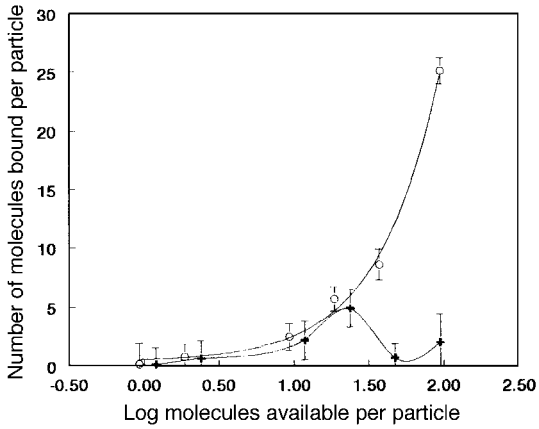

Fig. 4 Calculated number of hCG molecules bound at the surface of individual particles as a function of the log number of hCG molecules available per particle. The colloidal gold particles were coated, respectively, with two anti-hCG monoclonal antibodies (M2F05-094, open circles; and M2F03, crosses). The number of molecules bound was inferred from the difference in volume observed after incubation in presence of the ligand, the ligand molecule being considered as a sphere. The two antibodies are specific for a single epitope on the molecule.

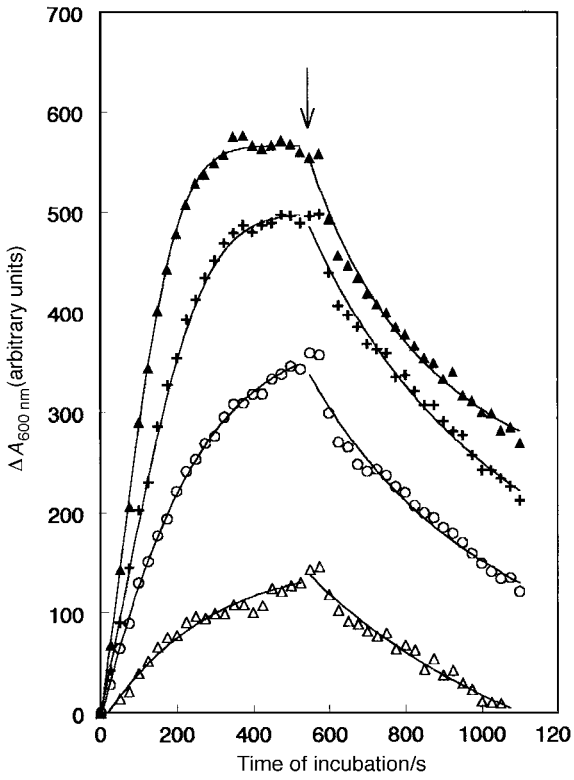

Fig. 5 Association and dissociation kinetics at 37 °C as measured in the Cobas-Mira instrument by the changes in *A* at 600 nm *versus* time using colloidal gold coated with anti-hFABP antibody 53E9. The association was monitored during incubation of the antibody with four ligand doses added in the cuvettes, respectively, 0.5 ng (open triangles), 0.9 ng (open circles), 1.9 ng (crosses) and 3.3 ng (closed triangles). The dissociation was induced by the addition of a 50 fold ligand excess (arrow).

Table 1 Comparison between the apparent affinity constants obtained from the automated colloidal gold SPR peak shift technology, Scatchard plots or BIAcore data, respectively, for various antibody–ligand interactions. The affinity constants obtained from the colloidal method are averages ± standard error of mean (SE) from two to five experiments performed each with different ligand doses. The correlation coefficients between data obtained from colloidal gold and from either Scatchard plot or the BIAcore are, respectively, 0.96 and 0.98

		Apparent affinity/mol^{-1}	
Antibody	Specificity	Comparative method	Automated colloidal gold method (± SE)
M2F05-057	β-hCG Epitope	2.0×10^{10} *	$1.6 \pm 0.4 \times 10^{10}$
M2F05-094	β-hCG Epitope	4.0×10^{10} *	$3.5 \pm 2.0 \times 10^{10}$
M2F03	α-β subunits joining Epitope of hCG	1.1×10^{10} *	$3.0 \pm 1.6 \times 10^{9}$
057-10010	Human liver ferritin	5.0×10^{9} *	$2.4 \pm 0.9 \times 10^{9}$
057-10030	Human spleen ferritin	4.0×10^{10} *	$3.1 \pm 1.0 \times 10^{10}$
090-10175	Human spleen ferritin	1.5×10^{11} *	$8.4 \pm 0.3 \times 10^{10}$
G5-F04	hCG α-Subunit	N.A.†	$7.0 \pm 1.6 \times 10^{9}$
53E9	hFABP Epitope	7.4×10^{7} ‡	$2.8 \pm 0.2 \times 10^{9}$
67D3	hFABP Epitope	3.4×10^{7} ‡	$3.4 \pm 0.7 \times 10^{9}$
72H10	hFABP Epitope	1.5×10^{8} ‡	$2.3 \pm 0.2 \times 10^{10}$

* Scatchard plot. ‡ BIAcore. ‡ N.A., not available.

tested being of 15 kDa (hFABP). The question now remains as to whether such technology is applicable to the interaction between receptors and smaller ligands.

I am indebted to Drs Hans-Georg Eisenwiener, Norbert Oranth and Carlos Salud (New Technologies Dept. of Hoffmann-La Roche Ltd, Basel) for the many interesting discussions and for the authorization to publish these experimental results.

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Paper 8/04010I
Accepted May 28, 1998