Mathematical Model of Simultaneous Diffusion and Binding of Antitumor Antibodies in Multicellular Human Tumor Spheroids

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ABSTRACT

Multicellular tumor spheroids are widely used as in vitro models of poorly vascularized tumor nodules in vivo. The uptake kinetics of tumor-associated antibodies in multicellular tumor spheroids is assumed to be governed by passive diffusion and irreversible binding of the antibodies with binding sites on the cell surface. By further assuming that the spheroids are homogeneous with respect to diffusion and binding, a mathematical model was developed which permits the extraction of the macroscopic diffusion constant \( D \) and the macroscopic binding rate \( k \) from empirical studies. The model was applied to uptake kinetics data obtained \( (\alpha) \) with a melanoma-associated monoclonal antibody 96.5 (isotype IgG2a)-human multicellular melanoma spheroid system exhibiting strong antibody to cell binding and \( (\beta) \) with the same monoclonal antibody-human multicellular colon adenocarcinoma HT29 spheroid system exhibiting nonspecific binding. The spheroids had approximately 300 \( \mu \)m diameter. The constants \( D \) and \( k \) were estimated to be 0.45 \( \mu \)m\(^2\)s\(^{-1}\) and 2.0 \( \times \) \( 10^{-3} \) s\(^{-1}\), respectively, for the system with specific binding. Saturation of binding sites occurred. In the nonspecific binding system, \( D \) and \( k \) were found to be 0.10 \( \mu \)m\(^2\)s\(^{-1}\) and 1.0 \( \times \) \( 10^{-5} \) s\(^{-1}\). No saturation of binding sites occurred. \( D \) and \( k \) were also estimated to be, respectively, 0.52 \( \mu \)m\(^2\)s\(^{-1}\) and 6.4 \( \times \) \( 10^{-9} \) s\(^{-1}\) for another melanoma-associated monoclonal antibody 140.240 (same isotype as 96.5) in the melanoma spheroid system exhibiting moderate cell binding with the antibody. The mathematical model describes well the system exhibiting nonspecific binding, but requires modifications and further development for the systems exhibiting moderate to strong binding.

INTRODUCTION

The uptake of MoAbs\(^3\) presented to solid tumors by the vascular system is determined by the physical processes of diffusion and binding. The macroscopic diffusion constant \( D \) and the first order irreversible binding constant \( k \) which appear in the partial differential equations describing diffusion with binding differ in general with tumor cell line and specific antibody. Determination of these fundamental constants of the process \( in vivo \) is prohibitively difficult because of a lack of control of the geometry of diffusion and of the concentration of antibody presented at the vascular system-tumor mass interface.

On the other hand, three dimensional spheroids grown from single tumor cells do permit control of both geometry and concentration. Spheroids consist of layers of actively replicating cells, inner layers of quiescent and nutrient deprived cells, and for sufficiently large spheroid radii, a core necrotic region. This growth pattern mimics the behavior of rapidly growing malignant solid tumors and provides an adequate experimental model of poorly vascularized tumor tissue.

In the present work, we develop solutions of the partial differential equations which describe the diffusion and binding of MoAbs by human multicellular tumor spheroids. For experimental protocols which include the use of radiolabeled MoAb in uptake studies we present a procedure for extracting both the diffusion constant and the binding constant. Finally, analytical expressions are obtained for the radial distribution of radiolabeled antibody as may be observed in autoradiographs of thin sectioned spheroids.

MATERIALS AND METHODS

Experimental Protocol

Uptake kinetics investigations were carried out with a mouse MoAb 96.5 (1) associated with human malignant melanoma in two types of human multicellular tumor spheroids, a melanoma and a colon adenocarcinoma (2), and with a mouse MoAb 140.240 (3, 4) in the melanoma spheroids. The two types of spheroids were grown from the human melanoma cell line CaCL 73-36 (5) and the human colon adenocarcinoma cell line HT29, which have been maintained in our laboratory since 1973. The MoAb 96.5 has high reactivity (binding equilibrium constant, \( 10^{9} \) M\(^{-1}\)) against the melanoma cells but only nonspecific binding with the colon cells. The MoAb 140.240 has moderate binding with the melanoma cells with equilibrium constant \(<10^{6} \) M\(^{-1}\) and nonspecific binding with the colon cells. Both MoAbs belong to the IgG2a isotype.

Groups of uniformly sized spheroids of 300 \( \mu \)m diameter were selected by sieving. They were incubated with \( ^{125}I \)-labeled antibody for 1, 4, 8, 11, 14, and 24 h at 37°C and approximately 0.2 ng/ml concentration. Details of the experiment have been described previously in another paper (2). Briefly speaking, about 50 spheroids sampled at each of the designated incubation times were washed briefly with phosphate buffered saline or reincubated with fresh culture medium without antibody for 1 or 24 h. These procedures were done to compare the short- and long-term retention of the antibody taken up by the spheroids. Amount of antibody effluxed during the reincubation process and the amount of antibody retained in the spheroids were measured.

Protocol for Theoretical Study

We first include a checklist of the notation used.

- **a**: Spheroidal radius (\( \mu \)m)
- **D**: Macroscopic diffusion constant (\( \mu \)m\(^2\)s\(^{-1}\))
- **k**: Reaction rate constant (s\(^{-1}\))
- **t\(_i\)**: Incubation time with radiolabeled antibody (s)
- **t\(_r\)**: Reincubation time in fresh medium (s)
- **C\(_i\)**: Concentration of radiolabeled antibody in incubation solution (cpm/ml)
- **C\(_r\)**: Concentration of radiolabeled antibody in reincubation solution (cpm/ml)

**Radial Distributions of Concentrations.**

- **L\(_i\)(r, t\(_i\))**: Unbound uptake after incubation (cpm/ml)
- **B\(_i\)(r, t\(_i\))**: Bound uptake after incubation (cpm/ml)
- **U\(_i\)(r, t\(_i\))**: Net uptake after incubation (cpm/ml)
- **L\(_r\)(r, t\(_r\))**: Unbound uptake after reincubation (cpm/ml)
- **B\(_r\)(r, t\(_r\))**: Bound uptake after reincubation (cpm/ml)
- **U\(_r\)(r, t\(_r\))**: Net uptake after reincubation (cpm/ml)

**Volume Integrated Concentrations per Spheroid.**

- **L\(_i\)(t\(_i\))**: Unbound uptake after incubation (cpm)
- **B\(_i\)(t\(_i\))**: Bound uptake after incubation (cpm)
- **U\(_i\)(t\(_i\))**: Net uptake after incubation (cpm)
- **L\(_r\)(t\(_r\))**: Unbound uptake after reincubation (cpm)
- **B\(_r\)(t\(_r\))**: Bound uptake during reincubation (cpm)

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2. To whom requests for reprints should be addressed.
3. The abbreviation used is: MoAb, monoclonal antibody.
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... Net uptake after reincubation (cpm)

\[ U_2(t) \] Concentration of radiolabeled antibody in reincubation solution (cpm/ml)

Spherical colonies of human malignant melanoma and HT29 cells were grown in culture to diameters in the range 300 to 350 \( \mu m \) as reported earlier (2). These spheroids were incubated for a time \( t_1 \) in a solution containing a known concentration \( C_i \) of \(^{125}\)I-labeled MoAb. Subsequently the spheroids were reincubated for a time \( t_2 \) in fresh culture medium with antibody concentration \( C_2 \) which was practically zero. The total uptake of the spheroids \( U_2(t_2) \) after reincubation and the total content of the reincubation solution \( W(t_2) \) were obtained by counting the activity of the radiolabeled antibody. Subsequently the spheroids were thin sectioned. Autoradiographs of the sections could be obtained. Quantitative analysis of the autoradiograph will be presented in a separate paper.

Other quantities of possible interest, for example the bound concentration resident in the spheroid after incubation, appear as intermediate results in the calculations detailed below. However, these quantities are not accessible to direct experimental observation in the present protocol.

**The Theory of Diffusion with Binding**

Before incubation, at time \( t = 0 \), the spheroids have zero initial concentration of MoAb, \( U_1(r, 0) = 0 \). The macroscopic diffusion constant \( D \) and the first order irreversible binding constant \( k \) are assumed to be independent of time and antibody concentration and distance from the spheroid center. These mathematical conditions are equivalent to the biological assumptions that (a) the binding sites are so numerous that saturation does not occur, and (b) that the spheroids are homogeneous and sufficiently small to exclude necrotic core regions.

Diffusion in a sphere without saturation of binding sites is governed by the following equations (6).

\[
\begin{align*}
1.1 & & \frac{\partial U_1}{\partial t} & = D \left( \frac{\partial^2 U_1}{\partial r^2} + \frac{2}{r} \frac{\partial U_1}{\partial r} \right) - kU_1 \\
1.2 & & U_1(r, 0) & = 0 \\
& & L_1(a, t_1) & = C_1 \\
& & L_2(a, t_2) & = C_2 \\
& & C_1 & = \text{constant} \\
& & D & = \text{constant} \\
& & k & = \text{constant}
\end{align*}
\]

Solution of these equations for both unbound and bound spheroid antibody concentration is developed in the Appendix.

The net uptake of antibody by a spheroid during the incubation period is the sum of the unbound and bound antibody concentrations

2. \[ U_1(t_1) = L_1(t_1) + B_1(t_1) \]

After reincubation the net antibody burden of the spheroid consists of the residual unbound concentration and the two bound concentrations acquired during both incubation and reincubation. This experimentally observable net concentration is

3. \[ U_2(t_2) = L_2(t_2) + B_1(t_1) + B_2(t_2) \]

Note that the concentration \( U_1(t_1) \) is observable only at time \( t_1 = 0 \). \( B_1 \) can be negative if the antibody becomes unbound. Finally, the experimentally accessible concentration of the reincubation solution containing the antibody washed out of the spheroids is simply the difference

4. \[ W(t_2) = U_1(t_1) - U_2(t_2) \]

The radial distribution after reincubation is also observable through autoradiography. This expression, an analogue of Equation 3, is

5. \[ U_2(r, t_2) = L_2(r, t_2) + B_1(r, t_1) + B_2(r, t_2) \]

In order to allow for possible saturation of binding sites in the present simple model, a parameter \( B_m \) representing the maximum binding site concentration is introduced so that \( B_1(r, t_1) + B_2(r, t_2) < B_m \). The parameter \( B_m \) is assumed to be a constant and to be independent of \( r \) because detailed variation of \( B_m \) with \( r \) was not available, although it is well known that spheroids are heterogeneous in structure. The number of binding sites for antibody 96.5 per CaCl 73-36 melanoma cell is of the order \( 2 \times 10^7 \) (3, 4) and there are of the order of \( 10^6 \) cells/ml in the melanoma spheroids. \( B_m \), therefore, is of the order of \( 3 \times 10^{-8} \) M.

**RESULTS**

Analytical Studies. The solutions of Equations 1.1 and 1.2 make it possible now to examine in detail the basic physical mechanisms of antibody diffusion with binding in a spherical cell colony.

First we study the incubation period during which antibody perfuses the spheroid by setting \( t_2 = 0 \). Experimentally, this corresponds to incubation followed by a very brief reincubation or rinse with a \( t_2 \) of about 5 s. This rinsing is carried out in order to dislodge any residual unbound antibody possibly adhering to the surface of the spheroid. A spheroid of radius \( a \) equal to 150 \( \mu m \) is incubated for \( t_1 = 3600 \) s in a solution containing the antibody at concentration \( C_1 \). This choice of spheroid radius is at the upper limit for cell viability at the core near \( r = 0 \) (7). Larger spheroids are expected to contain a necrotic core, for which alternate boundary conditions in the diffusion equations may be required.

The diffusion constant \( D \) for IgG molecules in wet tissues at 37°C, extrapolated from the data summarized by Swabb et al. (8), would be of the order 2 \( \mu m^2 \text{s}^{-1} \). A range of values of \( D \) from 0.5 to 3.5 \( \mu m^2 \text{s}^{-1} \) was therefore considered here. The dimensionless quantity \( U_2(t_2) = 0/C_1V \), where \( V \) is the volume of the spheroid, is shown on a log-log scale in Fig. 1 as \( k \) is varied. For \( ka^2/D \ll 1 \), reaction is slow compared with diffusion. The average concentration inside the spheroid after a 1-h incubation is less than that in the incubating solution. Here the removal of antibody from the unbound concentration within the spheroid is slow resulting in little enhancement in net uptake due to diffusion plus binding as opposed to diffusion alone. On the other hand, for \( ka^2/D \gg 1 \), reaction is fast compared with diffusion. The average concentration inside the spheroid is significantly enhanced. In this case antibody is rapidly removed from the unbound concentration inside the spheroid.

![Fig. 1. Ratio of average concentration (CONC.) of antibody (Ab) taken up by homogeneous spheroids of 300 \( \mu m \) diameter in 1 h to the concentration of the antibody in the incubation solution as predicted by the mathematical model for different reaction rate constants. Values of diffusion constant of the antibody in spheroids were assumed to be 0.5 (---), 2.0 (- - - -), and 3.5 (- - - -) \( \mu m^2 \text{s}^{-1} \).](image-url)
spheroid and a large gradient with a corresponding large flux is maintained across the surface of the sphere. For a constant $k = 5.5 \times 10^{-3} \text{ s}^{-1}$ which is typical for strong binding the variation of $U_2(t_1 = 0)/C_1$ as $D$ is varied is shown in Fig. 2. Here, for $D$ less than 1 $\mu\text{m}^2 \text{ s}^{-1}$, the rise in uptake with increasing $D$ is not rapid. This occurs since with $D$ small there is slow diffusion into the sphere and hence little antibody to bind in reaction. For $D$ approximately from 1 $\mu\text{m}^2 \text{ s}^{-1}$ to $k \cdot a^2$ (equal to 124 $\mu\text{m}^2 \text{ s}^{-1}$) however, increase of uptake with $D$ is most rapid. If $D$ for intact IgG molecules is indeed of the order 2 $\mu\text{m}^2 \text{ s}^{-1}$, the use of their fragments will significantly increase the uptake of the antibody. For large $D$ diffusion is rapid and increasingly supplies the binding sites with antibody. Eventually the reaction rate limits the uptake rate and the effect of increasing $D$ saturates.

An uptake versus incubation time $t_i$ with antibody curve $U_i(t_i)$ is presented in Fig. 3. $k$ is assumed to be $5 \times 10^{-4} \text{ s}^{-1}$. Since the number of binding sites in the present model is assumed infinite, there is again, as in the case of Fig. 1, no saturation, and uptake increases linearly with time for large $t_i$. At this moderate value of $k$, the ratio of the average concentration of antibody in the spheroid to that in the incubation medium is about 5 after 24 h of incubation.

Theoretical studies of the radial distributions of unbound and bound antibody are possible. With $D$ arbitrarily fixed at 0.5 $\mu\text{m}^2 \text{ s}^{-1}$ the variation of $L_4(r_2)/C_1$ with $r/a$ appears in Fig. 4. $t_i$ was assumed to be 1 h and $t_2 = 1$ s. Each curve corresponds to a value of the reaction rate. For the essentially zero value of $k = 5.0 \times 10^{-6} \text{ s}^{-1}$, the unbound concentration within the sphere approaches that of the incubation solution as an asymptote. The surface concentration is equal to that of the incubation solution. For increasing reaction rate, (a) the concentration of unbound antibody inside the sphere is everywhere reduced, and (b) there is a peaking introduced near the surface. Both effects are due to the binding of diffusing antibody. However, if $ka^2/D \gg 1$, more and more antibody is piling up at the surface. In this case, the rate of antibody uptake may be limited by the external mass transfer from the medium to the spheroid surface. Internal diffusion will be irrelevant.

The variation of $B_i(r_2)/C_1$ with $r/a$ is shown in Fig. 5 for the same range of $k$ values and same values for $t_1$ and $t_2$ as for Fig. 4. Note first the almost complete lack of binding for $k = 5.0 \times 10^{-6} \text{ s}^{-1}$. As $k$ increases, binding becomes significant, but with a pronounced peaking of antibody for values of $r$ near $a$. Since binding sites do not saturate, the unbound concentration near the surface is maintained low as in Fig. 4 and the concentration gradient across the surface is maintained high. Then there occurs a rapid diffusion across the boundary of the sphere accompanied by a rapid removal of free antibody through binding.

The observable radial distribution $U_i(r, t_1 = 1 \text{ h})$ of total diffusing antibody resident in the sphere is presented in Fig. 6.
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Fig. 6. Radial distribution of concentration (CONC.) of total antibody (Ab) (both bound and unbound) in spheroids of 300 μm diameter relative to the concentration of the antibody in the incubation solution as predicted by the mathematical model for the same four different reaction rate constants as in Fig. 4: A, 5 × 10⁻⁶; B, 5 × 10⁻⁵; C, 5 × 10⁻⁴; D, 5 × 10⁻³ s⁻¹. Incubation time = 1 h and diffusion constant = 0.5 μm² s⁻¹.

Table 1 Fitted values of diffusion constant D and reaction rate constant k for monoclonal antibody penetration in multicell tumor spheroids

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cell line</th>
<th>D (μm² s⁻¹)</th>
<th>k (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96.5</td>
<td>HT29</td>
<td>0.10</td>
<td>1.0 × 10⁻⁵</td>
</tr>
<tr>
<td>140.240</td>
<td>CaCL 73-36</td>
<td>0.35–0.52</td>
<td>3.3 × 10⁻⁴–6.4 × 10⁻⁴</td>
</tr>
<tr>
<td>96.5</td>
<td>CaCL 73-36</td>
<td>0.45</td>
<td>2.0 × 10⁻³</td>
</tr>
</tbody>
</table>

Fig. 7. Fitting of the mathematical model (—) over the Δ to measured uptake (O) of radiolabeled MoAb 96.5 by HT29 spheroids of 300 μm diameter. Bars, 1 SE. The spheroids had a 1-h wash with culture medium without MoAb after the specified times of incubation with MoAb.

Fig. 8. Fitting of the mathematical model to the three earlier (— — —) or all (—— — —) the time points of measured uptake (Δ) of radiolabeled MoAb 140.240 by the melanoma spheroids of 300 μm diameter. Bars, 1 SE. The spheroids had a 1-h wash with culture medium without MoAb after the specified times of incubation with MoAb.

This net spheroid burden of antibody is dominated by the bound term contribution with its attendant surface peaking. It suggests that a poorly vascularized region of tumor may exhibit a highly nonuniform uptake of antibody throughout its volume if the antibody is strongly bound. Delivery to the more active cells near branches of the vascular system is enhanced. Tumor regions containing quiescent cells remain essentially barren of antibody.

Experimental Data Analysis. As a test of the applicability of the mathematical model of diffusion with binding developed above, we have fitted Equations 3 and 4 to experimental data with t₁ = 1, 4, 8, 11, 14, and 24 h and t₂ = 1 h by identifying values of D and k for which χ² is minimized. Results are summarized in Table 1.

Antibody 96.5 uptake by the HT29 spheroids with D equal to 0.10 μm² s⁻¹ and k equal to 1.0 × 10⁻⁵ s⁻¹, at which minimum χ² occurred, is shown in Fig. 7. Agreement of computed values, denoted by — — — and experimental data, denoted by the circles with ± one SE bar, is good. In this system both diffusion of antibody into the tumor spheroid and binding to tumor cells are low resulting in a low antibody concentration resident in the spheroids after incubation.

The continuous curve in Fig. 8 shows the minimum χ² fitting of antibody 140.240 uptake by the melanoma spheroids to experimental data. D was found equal to 0.35 μm² s⁻¹ and k equal to 3.3 × 10⁻⁵ s⁻¹. Agreement between experimental and fitted values is not as good as in Fig. 7 because of some degree of saturation and/or shedding of the MoAb occurring in the 140.240 and melanoma spheroid system by 12 h. When the model was applied to the time points shorter than 12 h, the minimum χ² fit resulted in D equal to 0.52 μm² s⁻¹ and k equal to 6.4 × 10⁻³ s⁻¹. This fitting is represented by — — — in Fig. 8.

In contrast, a least-squares fit to antibody 96.5 uptake by the melanoma spheroids yielding D and k equal to 0.16 μm² s⁻¹ and 2.0 × 10⁻³ s⁻¹, respectively, is represented by — — — in Fig. 9. The fitting is poor due to saturation of binding sites from about 11 h onward. This system shows a binding rate constant two orders of magnitude greater than the preceding cases. The antibody uptake rate is now bound by the antibody available through the process of diffusion. Better fitting (as represented by — — —) to the data is achieved when the maximum binding site concentration Bₘ equal to 3 × 10⁻⁸ M was introduced as the third fitting parameter. D and k are concomitantly changed to 0.25 μm² s⁻¹ and 2.5 × 10⁻³ s⁻¹, respectively. Best fitting (as represented by — — —) was obtained at k, D, and Bₘ, respectively, equal to 2 × 10⁻³ s⁻¹, 0.45 μm² s⁻¹ and 2.5 × 10⁻⁸ M. Note that k does not vary and the change in D is about 3-fold compared with the two-parameter k, D fitting. Possible reasons for the failure of the simple model to give good fitting to melanoma spheroids include saturation of binding sites, shedding of antibody-antigen complexes from the cell surface, and nonuniform values of D and k with respect to radial distance in the spheroids.
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**DISCUSSION**

The macroscopic binding rate constant \( k \) is equal to the product of concentration of binding sites, \([Ag]\), and the forward rate constant \( k_1 \) of the following reversible molecular reaction

\[
Ab + Ag \xrightleftharpoons{\k_1}{\k_2} Ab - Ag
\]

where \( Ab \) stands for the antibody and \( k_2 \) the reverse rate constant. For the antibody 96.5 and CaCl\(_2\) 73-36 melanoma cells at neutral pH, \( k_1 \) is of the order \( 5 \times 10^{-7} \text{ s}^{-1} \text{ M}^{-1} \) (9, 10) and \( [Ag] \) of the order \( 3 \times 10^{-8} \text{ M} \) (see the derivation of \( D_m \) under "Theory of Diffusion with Binding"), resulting in \( k \) of the order \( 1.7 \times 10^{-3} \text{ s}^{-1} \). This sets the upper limit of \( k \) and is in good agreement with our estimated value of \( 2.0 \times 10^{-3} \text{ s}^{-1} \) for the melanoma spheroids. Complete saturation of binding sites in the 300-\( \mu \text{m} \) diameter melanoma spheroids occurred at about 14 h in the experiments (Fig. 9).

It is instructive to examine the radial distributions of antibody after incubation with the antibody for 24 h in the three systems as estimated by Equation 5 with the fitted values of \( D \) and \( k \) of Table 1. These uptake distributions are shown on a semilogarithmic scale in Fig. 10. Experiments are being performed to obtain these distributions as another check on the usefulness of the mathematical model.

The 140.240-melanoma system in comparison to the nonspecific 96.5-HT29 system has a greater antibody uptake as expected. In addition, the greater value of \( D \) alters the radial distribution, enhancing the concentration of antibody near the core of the spheroid. It is of interest to point out that the \( D \) values of the antibodies 140.240 and 96.5 in the melanoma spheroids are similar in magnitude and are about 4-fold as great as that for antibody 96.5 in the HT29 spheroids. This agrees with the observation that the HT29 spheroids are more compact in structure than the melanoma spheroids from histological study of the spheroids (data not shown).

The significantly larger reaction rate in the 96.5-melanoma system binds free antibody so rapidly near the surface of the spheroid that diffusion into the core region is inhibited. In fact, concentrations near the center in the 96.5-melanoma spheroids are lower than in the two systems with weaker binding. This effect becomes significant in some tumor dose calculations for antibodies conjugated with either short-ranged radionuclides or other cytotoxic agents.

Based on published values of diffusion coefficient \( D_{im} \) of solutes of a wide range of molecular weights in tissue up to 1974, Swabb et al. (8) presented an empirical relationship between the molecular weight and \( D_{im} \) for molecular weight less than 70,000. If this relationship is assumed valid for IgG molecules, then the corresponding \( D_{im} \) will be about \( 2.3 \mu \text{m}^2 \text{ s}^{-1} \) at 37°C. This value is about 5-fold as large as the \( D \) value for MoAb 96.5 in the melanoma spheroids obtained here. In the same reference, Swabb et al. (8) further suggested that the transport of IgG molecules in tissue is likely to be dominated by convection. Whether convection is important or not in the transport of IgG molecules in small tumor nodules, especially when there is negligible difference in the interstitial fluid hydrostatic pressure between the surface and the center of the nodules, remains an open question.

**APPENDIX**

Solution of Equations 1.1 and 1.2 during the incubation proceeds (11) by first setting \( k = 0 \) and making the substitution \( S = L_1 \) in order to obtain an expression for the unbound concentration \( L_1(r, t) \) in the spheroid resulting from diffusion without reaction. Defining \( \delta = Dn^2/r^2 \) \n
\[ A_1. \quad L_1(r, t) = C_1 + C_1 \frac{2a}{\pi r} \sum \frac{(-1)^i}{n} \left[ \sin \frac{n \pi r}{a} \right] \exp(-bt_i) \]

This expression is then used with Danckwerts' method (6) for the solution for diffusion with reaction

\[ A_2. \quad L_1(r, t) = C_1 + C_1 \frac{2a}{\pi r} \sum \frac{(-1)^i}{n} \left[ \sin \frac{n \pi r}{a} \right] \left\{ \frac{k + \delta \exp[-(k + \delta)t_i]}{k + \delta} \right\} \]

The bound concentration of diffusing antibody may be calculated by the integral

\[ A_3. \quad B_1(r, t) = k \int_0^t L_1(r, t) \, dt \]

This evaluates to

\[ A_4. \quad B_1(r, t) = kt_1C_1 + kt_2C_2 \frac{2a}{\pi r} \sum \frac{(-1)^i}{n} \left[ \sin \frac{n \pi r}{a} \right] \left( \frac{k}{k + \delta} \right) \]

Diffusion with reaction during reincubation is also described by Equation 1.1 under "Materials and Methods" but with a more general set of boundary conditions. These are that there is an initial nonzero and nonuniform distribution \( g(r) \) of unbound antibody resident in the spheroid at the beginning of reincubation and that the reincubation solution concentration \( C_2 \) may be a function \( h \) of time \( t_s \), i.e.,

\[ A_5. \quad U_1(r, 0) = g(r) \]

\[ C_2(t_s) = h(t_s) \]

Utilizing the same substitutions as above, equations 1.1 and A5 have a solution (12)

\[ A_6. \quad L_1(r, t_s) = \frac{2}{ra} \sum \frac{(-1)^i}{n} \left[ \sin \frac{n \pi r}{a} \right] \exp[-(k + \delta)t_s] \]

\[ \int_0^t dt' \, g(t') \sin \frac{n \pi r}{a} \]

for reincubation of spheroids in an infinite solution where \( C_2(t_s) \) is held to zero.

The initial distribution required is just the unbound concentration at
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the termination of incubation, equation A2. Substitution into equation A6 yields

\[ A7. \quad L_2(r, t_2) = \frac{2aC_1}{\pi r} \sum_{n=1}^{\infty} (-1)^n \left( \frac{\sin(n\pi r)}{n} \right) \exp[-(k + \delta)t_2] \left\{ \frac{k + \delta \exp[-(k + \delta)t_1]}{k + \delta} - 1 \right\} \]

Again there is a further binding during reincubation while antibody diffuses out of the spheroid into the washing solution. After integration analogous to A3 above, we obtain

\[ A8. \quad B_2(r, t_2) = \frac{2aC_1}{\pi r} \sum_{n=1}^{\infty} (-1)^n \left( \frac{\sin(n\pi r)}{n} \right) \left( \frac{k}{k + \delta} \right) \left\{ 1 - \exp[-(k + \delta)t_2] \right\} \left\{ \frac{k + \delta \exp[-(k + \delta)t_1]}{k + \delta} - 1 \right\} \]

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