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BRIEF REPORT

Osmotic Response of Lymphocytes Measured by Means of Forward Light Scattering: Theoretical Considerations[1,2]

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Recent data provide solid experimental evidence that increase in cell size of human lymphocytes, caused by osmotic stress, is not directly proportional to forward scattering intensity but rather is inversely proportional. Here, we provide arguments that this phenomenon can be quantitatively explained by means of a model based on a modified Rayleigh-Debye-Gans theory. As a consequence, the current view that forward scattering intensities can be used as a measure of gross cell size needs to be reconsidered. In addition, we postulate that structural changes of the cytoplasm, imposed by an osmotic stress, reflect corresponding changes in the nucleus.

Key terms: Concentric sphere model, flow cytometry, osmosis

Regulation of cell volume following exposure to anisotonic media has been extensively studied to improve our understanding of the mechanisms employed by a cell to adapt to anisotonic situations. For instance, basic research concerning the mechanisms involved in cellular response to extreme osmotic pressure was reported recently (13). Knowledge of these regulating mechanisms can be exploited to characterize various subpopulations of human lymphocytes. It was reported that identification of human peripheral B and T lymphocytes is facilitated by means of their differences in cell volume regulation after a hypotonic shock (6). Corresponding techniques can also be applied to study the cell lineage of lymphoid leukemia cells (9) or the dynamic behavior of various normal and leukemic lymphocytes (3). Apart from the influence of anisotonic conditions on the gross cell size, influence on the nucleus of human cultured cells (EUE-cell line), or more generally, freshly isolated human leukocytes has been studied (8,19).

Recently, McGann et al. measured simultaneously changes in the forward light scattering intensities and the electronically sized cell volumes of lymphocytes following an anisotonic shock (14). They showed unambiguously that forward scattering (FS) intensities vary inversely with cell volume. This paradoxical result could not be explained, since low-angle light scattering is assumed to be proportional to cellular size (11,12,15,26). These results prompted us to investigate theoretically and in detail the light scattering phenomena of human lymphocytes measured under various osmotic conditions.

MATERIALS AND METHODS

Light Scattering From Lymphocytes

The field scattered by a particle that is large compared to the wavelength of the light illuminating it can be approximately subdivided into diffraction, refraction, and reflection components (28). It can be shown that diffraction predominates for scattering angles up to $\theta_{max} = 1.22\ \lambda/d$ [rad] (4) where λ is the wavelength of the incident light and d is the diameter of the particle. Diffraction effects are essentially independent of refractive index. Therefore, the detection of light scattered in the first FS lobe (for particles with a diameter of 7 μm and λ = 488 nm, this lobe is confined to $\theta_{max} = 4.9°$) has been used as a measure for cellular size (11,12,18, 22,24,26,27).

In a previous paper, we discussed a model to describe elastic light scattering from nucleated blood cells (25). In this model, a nucleated blood cell (e.g., a lymphocyte) is modeled by two concentric spheres. The inner sphere denotes the nucleus, and the outer shell defines the cytoplasmatic region (Fig. 1). The light scattering characteristics were derived from the Rayleigh-Debye-Gans approximation and averaging of the internal field. In

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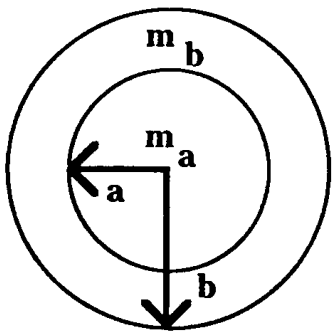

FIG. 1. A nucleated blood cell modeled by two concentric spheres. m_a, m_b = relative refractive indices; a, b = radii of concentric spheres.

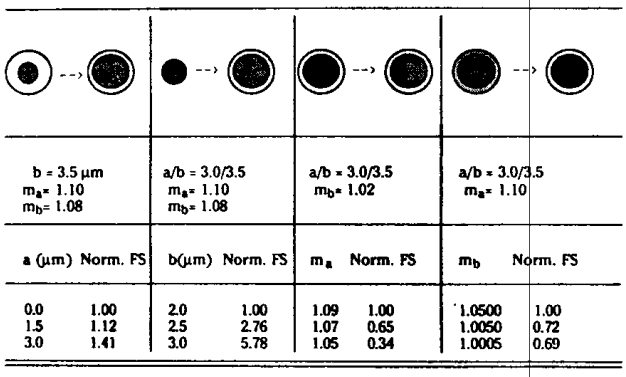

FIG. 2. Influence of changes in scatter parameters on the normalized forward scattering intensities from Sloot et al. (25).

addition, modification of the wave number inside the different cellular compartments was required to facilitate calculations for particles as large as human peripheral lymphocytes (~7 μm) (25). It was shown that the angular intensity of the scattered radiation $I(\theta)$ was proportional to the form factor $P(\theta)^2$ where

$$P(\theta) = (2\pi)^{3/2}\left(\alpha'_a \frac{a^3}{u_1^{3/2}} J_{3/2}(u_1) + \alpha'_b\left[\frac{b^3}{u_2^{3/2}} J_{3/2}(u_2) - \frac{a^3}{u_3^{3/2}} J_{3/2}(u_3)\right]\right), \quad (1)$$

where $u_1 = 2ak_0m_a \cdot \sin(\theta/2)$, $u_2 = 2bk_0m_b \cdot \sin(\theta/2)$, and $u_3 = 2ak_0m_b \cdot \sin(\theta/2)$. α'_a and α'_b are the volume polarizabilities (25,28) of the nucleus and the cytoplasm, respectively, and $J_{3/2}(u)$ represents the three half-order Bessel function (1). The wave number k_o is $2\pi/\lambda_o$, where λ_o is the wavelength of the incident light in vacuum.

These equations can be applied to calculate the complete scattering spectrum of lymphocytes given the various parameters. In Figure 2 we summarize the influence of small variations in size and relative refractive indices (a, b, m_a, and m_b) on the forward light scattering intensities. The incident light is a He/Ne laser (λ = 632.8 nm), and the cells are irradiated in an aqueous solution (refractive index = 1.333). The calculations apply for parallel polarized incident light. The FS intensities were integrated from 1° to 7°. Further details concerning these calculations are described elsewhere (25).

It can be derived from Figure 2 that FS is proportional to changes in the nuclear size or the gross cell size. This result is consistent with the idea that FS increases with increasing cellular size. However, a decrease in refractive index of the nucleus or the cytoplasm results in a corresponding decrease of the FS signal. These data suggest that the influence of changes in the refractive index of both the cytoplasm and the nucleus should be considered when FS of nucleated blood cells is concerned.

Proposed Model

In this section we examine the physical consequences of the osmotic response of cells in an anisotonic environment.

Basically, there are three diffusion mechanisms that allow a cell to adjust to anisotonic media: free diffusion, facilitated diffusion, and active diffusion (7). It has been shown by many authors that, to a first approximation, the dynamics of diffusion are well described by free diffusion (3,10,13,14,23). In the case of free (or passive) diffusion, the condition for equilibrium requires that the chemical potential in the cell equals the chemical potential in the medium. The volume of the cell V_{cell} equals the volume of the intracellular water (V) plus the volume occupied by the endogenous cell solutes V′ (13). Therefore, the part $(V_{cell} - V') = V$ contributes dynamically to the equilibrium condition. From thermodynamical considerations the response of a lymphocyte, for example, to an osmotic pressure Π will be (16)

$$\int_0^{\Pi} V\, dP = -RT \ln \frac{P}{P^*}, \quad (2)$$

where P/P^* (= X) indicates the relative vapor pressure of the solvent divided by the vapor pressure of the pure solvent, R is the gas-constant ($JK^{-1}mol^{-1}$), and T is the temperature (°K). This results in

$$V\,\Pi = -RT \ln X \approx RT(1-X). \quad (3)$$

Assuming mass conservation of the cell solutes we obtain

$$V_{cell} = \frac{\Pi^\circ}{\Pi}(V^\circ_{cell} - V') + V', \quad (4)$$

where the isotonic situation is labeled by Π° and V°_{cell}. This alternative derivation of the Boyle van 't Hoff

equation results directly in a linear relationship of the cellular volume V_{cell} vs. the changes in osmotic pressure Π°/Π.

In the previous section we modeled a lymphocyte by two concentric spheres. In addition it is assumed that both the inner and the outer sphere act like an ideal osmometer. Eventually, the changes in cellular/nuclear volume can be derived from equation 4. Next, the influence of changes in refractive index of the two compartments involved are investigated. It is assumed that solutes neither enter nor leave the cell (13). Since the refractive indices are related to the number of dipoles per volume, changes in volume will induce changes in refractive index. Quantification of this phenomenon can be obtained from the Lorenz-Lorentz equation (25,28):

$$\frac{m^2 - 1}{m^2 + 2} = C\frac{1}{V}, \tag{5}$$

where C [m^3] is a constant. Taylor expansion results in (assuming $m - 1 \rightarrow 0$)

$$m - 1 = \frac{3C}{2V}, \tag{6}$$

The volume V can be calculated from equation 4.

Consequently, the influence of the application of an osmotic shock on the scattering parameters m_a, m_b, a, and b is obtained from

$$\begin{aligned} m &= \frac{m_o - 1}{\Pi^\circ/\Pi} + 1 \\ b &= b^\circ\left(\frac{\Pi^\circ}{\Pi}\right)^{1/3} \\ a &= b\left(\frac{3}{3.5}\right), \end{aligned} \tag{7}$$

where it is assumed that $V' \ll V^\circ_{cell}$.

This simultaneous increase in volume and decrease in refractive index is illustrated in Figure 3. The ratio nucleus/cytoplasm, 3/3.5, is used as a typical value for human peripheral lymphocytes (2,25).

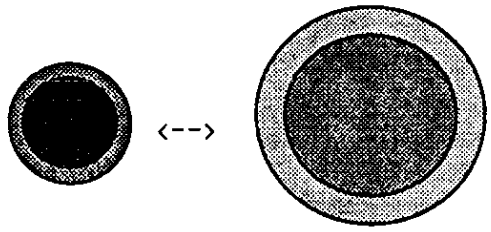

FIG. 3. Illustration of an osmotic shock on the scattering parameters a, b, m_a, and m_b of a nucleated blood cell. The darker areas denote a relatively higher refractive index.

In the next section, this model is evaluated to calculate the changes in the FS intensities of light scattered from a lymphocyte in various anisotonic solutions.

RESULTS

Measurement of cell volumes in osmotically stressed and frozen-thawed cells was recently reported by McGann et al. (14). Their Figure 1a shows the influence of both the electronically sized volume changes and the FS intensities vs. Π°/Π. The light scattering experiments were performed by means of an EPICS V flow cytometer with an incident wavelength of 488 nm and an effective acceptance angle of 2.2° to 6.2°. Changes in osmolarity from $\Pi^\circ/\Pi = 0.4$ to $\Pi^\circ/\Pi = 1.6$ (1.0 = isotonic) were recorded. We calculated the corresponding changes in scattering parameters, for this range of osmolarities, in accordance with the equations derived in the previous section. The resulting values are tabulated in Table 1.

Next, equation 1 is numerically integrated with the defined acceptance angle, by means of the trapezoidal rule (1):

$$FS = \int_{2^\circ}^{6^\circ} P(\theta)\sin(\theta)\,d\theta.$$

(Note that the range of integration corresponds to the experimental set-up of McGann et al. (14) and differs from that applied in reference 25). The variables of Table 1 are inserted in the form factor $P(\theta)$ (step-size $\Delta(V/V^\circ) = 0.06$), the result of which is shown in Figure 4. The experimental values from McGann et al. (14) are shown in Figure 5.

Finally, the data of both figures were fit to an exponential curve. This resulted in: Norm. FS = $2.16 \cdot 10^{(-0.33\ \Pi^\circ/\Pi)}$ for the theoretical calculations (Fig. 4) and Norm. FS = $2.31 \cdot 10^{(-0.38\ \Pi^\circ/\Pi)}$ for the experimentally observed intensities (Fig. 5). The correlation coefficients are 0.99 and 0.97, respectively.

DISCUSSION

Interpretation of low-angle light scattering from particles as large as biological cells is usually based on the Fraunhofer diffraction theory (5,15,29). Here it is assumed that light passing along a particle forms a plane wavefront, which can be divided into a number of indi-

Table 1
Changes in Light Scattering Parameters for a Lymphocyte Modeled by Two Concentric Spheres, in Anisotonic Solutions

	Environment		
	Hypertonic	Isotonic	Hypotonic
$V/V^\circ = \Pi^\circ/\Pi$	0.40	1.00	1.60
a (μm)	1.90	2.57	3.01
b (μm)	2.21	3.00	3.51
m_a	1.25	1.10	1.06
m_b	1.13	1.05	1.03

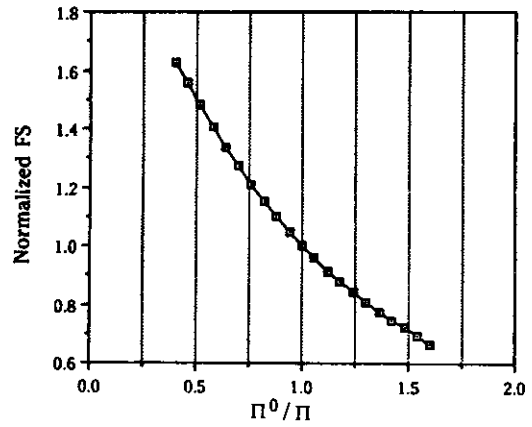

FIG. 4. Implementation of the proposed model and numerical integration of the form factor $P(\theta)$. Π = Osmotic pressure; Π° = osmotic pressure for the isotonic situation. The forward scattering (FS) intensities are normalized to unity for cells in an isotonic environment.

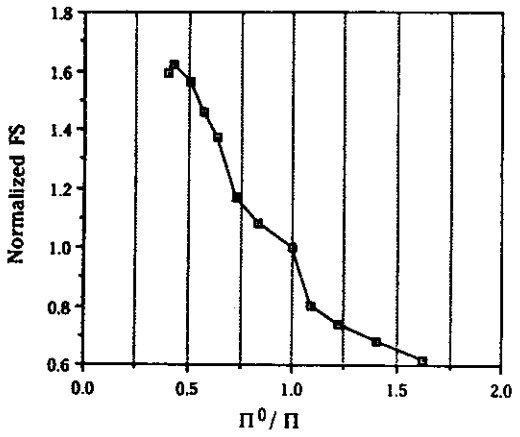

FIG. 5. Measurement of forward light scattering from osmotically stressed lymphocytes (ref. 14). Π = Osmotic pressure; Π° = osmotic pressure for the isotonic situation. The forward scattering (FS) intensities are normalized to unity for cells in an isotonic environment.

vidual rays, following the Huygens principle (4). As a consequence, FS can be regarded as a projected area response, and no information on cellular transparency is present. It was shown by Steinkamp et al. (27) that for *homogeneous* particles, varying in size, but not in refractive index, a proportionality exists between FS and electronically sized uniform plastic microspheres. The influence of beamshaping optics on the linearity of the FS signal vs. the volume was discussed both theoretically and experimentally (27,30). From these observations it can be concluded that, to a first approximation, FS is proportional to d^x where $1 \leqslant x \leqslant 3$ and d is the diameter of the particle. The exact value of x is a complex function of cellular size, optics, detection system, and range of detection angles. However, the data from Figure 2 clearly indicate that changes in refractive index for nucleated blood cells (i.e., lymphocytes) introduce an inverse effect on the FS intensities. Therefore, to investigate the phenomenon of FS decrease with increasing cellular size under hypotonic stress, we suggest the model described in the preceding sections. A number of assumptions are implicit here.

First, it is assumed that both the gross cell size and the size of the nucleus progressively change when the cells are osmotically stressed. From the Coulter volume data reported by McGann et al. (14), it is obvious that the cytoplasm swells in hypotonic media and shrinks in hypertonic media. It has been reported, however, that, because of the large pores in the nuclear membrane, *isolated* nuclei do not swell in hypotonic media (21). Experiments with *intact* mammalian cells, however, indicate that nuclei of human lymphocytes, monocytes and granulocytes show conformational changes in a hypotonic environment (8,17,19). We postulate, as a possible explanation to this antinomy, that the fibrillar networks inside the cell regulate the changes in the size of the nuclei. Poste (20), for instance, reports that cellular cytoskeletal structures play a pivotal role in regulating shape, deformability, and contraction of the cell and nucleus.

Figure 6 illustrates 1) the influence of changes in the refractive index of the cytoplasm, apart from changes in the refractive index of the nucleus and 2) changes in the refractive index of the nucleus with changes imposed on the parameters of the cytoplasm. From this two-step process, it can be derived that the "inverse phenomenon" is qualitatively explained by the decrease in refractive index of the cytoplasm, whereas the decrease in refractive index of the nucleus results in a quantitative correspondence between the experimental data and the proposed model (the beginning and ending values of Fig. 6 agree with the corresponding values of Fig. 4). These findings support the notion that, although the nucleus

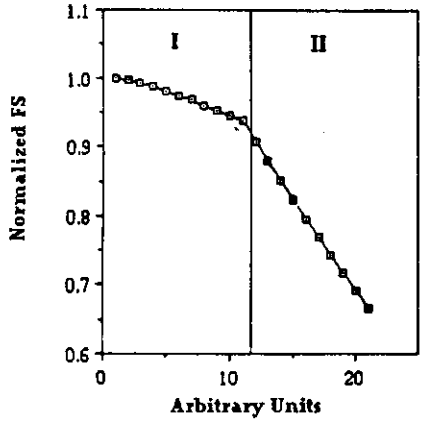

FIG. 6. Relative influence of (I) increase in cellular size and corresponding decrease in refractive index of the cytoplasm; (II) increase in nuclear size, corresponding decrease in the refractive index of the nucleus, and simultaneous increase in the refractive index of the cytoplasm. I: $a = 2.571$; $3.00 \leqslant b \leqslant 3.509$; $m_a = 1.100$; $1.05 \geqslant m_b \geqslant 1.019$. II: $2.571 \leqslant a \leqslant 3.007$; $b = 3.509$; $1.100 \geqslant m_a = 1.064$; $1.019 \leqslant m_b \leqslant 1.032$.

of a mammalian cell does not behave as an ideal osmometer, the conformational changes in the size and refractive index can be modeled by the relations proposed in the preceding sections.

A second assumption is that the refractive index of the environment remains constant within the ranges of osmolarities observed. Measurement of polystyrene particles in the various anisotonic solutions revealed no influence on the FS intensities. It can be concluded that, within the accuracy of the measurement, no changes in refractive index of the medium is expected.

Finally, the assumption that the relative refractive index changes can be derived from equations 6 and 7 is true only if $m \rightarrow 1$. Table 1 shows that for extreme hypertonic situations, this approximation is not valid anymore. However, direct insertion of the Lorenz-Lorentz equation (eq. 5) instead of the expansion (eq. 6) into the calculations for hypertonically stressed lymphocytes showed a close resemblance to the data obtained in the previous section. It was concluded, therefore, that a re-[e]nement of equation 6 within the range of observed .nolarities does not contribute to our model. In addition, small changes in the ratio of nucleus/cytoplasm (3/3.5), changes in the range of size parameters or refractive index, or small changes in the span of integration angles did not affect our results significantly (data not shown).

CONCLUSIONS

We have provided a theoretical basis to explain the apparent forward light scattering anomalies of osmotically stressed lymphocytes measured in a flow-through system. It was shown that a simple light scattering algorithm can be applied to monitor changes in cellular morphology. The proposed model describes the changes in refractive index of the cellular compartments and, simultaneously, the changes in the size of the nucleus and the cytoplasm for lymphocytes in anisotonic solutions. The theoretical data correspond quantitatively with the experimentally obtained data. As a conse-[q]uence, the conventional interpretation of forward light [s]cattering phenomena, especially in flow cytometric measurements, needs to be reconsidered.

To the best of our knowledge, this study describes for the first time quantitatively the correspondence between theoretical and experimental light scattering data of morphological changes in living cellular material measured in a flow cytometer.

The hypothesis that size changes in the cytoplasmatic region of the cell reflect corresponding changes in the nucleus has not been verified extensively. In the near future, we will study this hypothesis in more detail. Preliminary measurements of the nuclear size of osmotically stressed lymphocytes confirm the suggested volume response.

In addition to previous work, in which the lateral and back scattering directions for concentric spheres were investigated (25), the results presented here legitimate a further detailed study of the light scattering phenomena of biological cells that can be described by the form factor $P(\theta)$. For instance, extrapolation of the form factor to describe more complex cellular structures will be explored, and detailed cellular texture such as granules and segmented nuclei may be studied numerically.

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